



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>A61K 31/00, 35/00, 38/00, C07K 14/435, 14/705, C12N 5/10, 15/12, 15/63, G01N 33/53, 33/566</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 98/18456</b> <b>(43) International Publication Date:</b> 7 May 1998 (07.05.98)
<b>(21) International Application Number:</b> PCT/US97/19732 <b>(22) International Filing Date:</b> 29 October 1997 (29.10.97) <b>(30) Priority Data:</b> 08/742,440 30 October 1996 (30.10.96) US <b>(71) Applicant:</b> THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 22nd floor, 300 Lakeside Drive, Oakland, CA 94612-3550 (US). <b>(72) Inventors:</b> COUGHLIN, Shaun, R.; 2 Turtle Rock Court, Tiburon, CA 94920 (US). ISHIHARA, Hiroaki; 1770 17th Avenue, San Francisco, CA 94122 (US). CONNOLLY, Andrew; 1427 Schrader Street, San Francisco, CA 94117 (US). <b>(74) Agent:</b> BERLINER, Robert; Fulbright & Jaworski, L.L.P., 29th floor, 865 South Figueroa Street, Los Angeles, CA 90017-2571 (US).		<b>(81) Designated States:</b> CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>

**(54) Title:** PROTEASE-ACTIVATED RECEPTOR 3 AND USES THEREOF**(57) Abstract**

Disclosed are cDNAs and genomic DNAs encoding protease-activated receptor 3 (PAR3) from mouse and human, and the recombinant polypeptides expressed from such cDNAs. The recombinant receptor polypeptides, receptor fragments and analogs expressed on the surface of cells are used in methods of screening candidate compounds for their ability to act as agonists or antagonists to the effects of interaction between thrombin and PAR3. Agonists are used as the therapeutics to treat wounds, thrombosis, atherosclerosis, restenosis, inflammation, and other thrombin-activated disorders. Antagonists are used as therapeutics to control blood coagulation and thereby treating heart attack and stroke. Antagonists mediate inflammatory and proliferative responses to injury as occur in normal wound healing and variety of diseases including atherosclerosis, restenosis, pulmonary inflammation (ARDS) and glomerulosclerosis. Antibodies specific for a protease-activated receptor 3 (or receptor fragment or analog) and their use as a therapeutic are also disclosed.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

- 1 -

## PROTEASE-ACTIVATED RECEPTOR 3 AND USES THEREOF

## Field of the Invention

This invention relates to nucleic acids, their  
5 encoded protease-activated receptor 3 proteins, and  
screening assays for agonists and antagonists of the  
protease activated receptor 3 proteins.

## Background of the Invention

Thrombin, a coagulation protease generated at  
10 sites of vascular injury, activates platelets,  
leukocytes, and mesenchymal cells (Vu, T.-K.H. et al.  
(1991) Cell 64:1057-1068). Activation of platelets by  
thrombin is thought to be critical for hemostasis and  
thrombosis. In animal models, thrombin inhibitors block  
15 platelet-dependent thrombosis, which is the cause of most  
heart attacks and strokes in humans. Available data in  
humans suggests that thrombosis in arteries can be  
blocked by inhibitors of platelet function and by  
thrombin inhibitors. Thus it is likely that thrombin's  
20 actions on platelets contribute to the formation of clots  
that cause heart attack and stroke. Thrombin's other  
actions on vascular endothelial cells and smooth muscle  
cells, leukocytes, and fibroblasts may mediate  
inflammatory and proliferative responses to injury, as  
25 occur in normal wound healing and a variety of diseases  
(atherosclerosis, restenosis, pulmonary inflammation  
(ARDS), glomerulosclerosis, etc.). A thorough

- 2 -

A receptor that mediates thrombin signaling has been previously identified (Vu, T.-K.H. et al. (1991) Cell 64:1057-1068; USPN 5,256,766). This receptor revealed a novel proteolytic mechanism of activation and is referred to as PAR1 (protease-activated receptor 1). PAR1 is activated by the binding of thrombin to and cleavage of PAR1's amino terminal exodomain at a specific site. Receptor cleavage unmasks a new amino terminus, which then functions as a tethered peptide ligand by binding intramolecularly to the body of the receptor to effect transmembrane signaling (Vu, T.-K.H. et al. (1991) Cell 64:1057-1068). Synthetic peptides that mimic this tethered ligand domain function as PAR1 agonists and activate it independent of thrombin and receptor cleavage (Vu, T.-K.H. et al. (1991) Cell 64:1057-1068).

To identify which of thrombin's known cellular actions are mediated by PAR1, a PAR1 knockout mouse was recently generated (Connolly, A. et al. (1996) Nature 381:516-519). Analysis of mice in which both alleles of the PAR1 gene were disrupted provided definitive evidence for a second platelet thrombin receptor and for tissue specific roles of distinct thrombin receptors. Specifically, in mice, PAR1 is not important for platelet responses but is critical for fibroblast responses.

A second protease-activated receptor (PAR2) was cloned during a search for relatives of the Substance K receptor (Nystedt, S., et al. (1994) PNAS USA, 91:9208-9212). The physiological activator of PAR2 remains unknown; it is not activated by thrombin.

30

## SUMMARY OF THE INVENTION

The protease-activated receptor (PAR3) disclosed herein is useful in assaying libraries of compounds for their activity as thrombin agonists and antagonists. DNA

- 3 -

encoding PAR3 is placed in a functional expression vector, expressed in a cell line, and used to assay compounds for activity as an agonist or antagonist of thrombin's affect on PAR3.

5           The invention features substantially pure DNA (cDNA or genomic DNA) encoding a protease-activated receptor 3 (PAR3) from vertebrate tissues (SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:5) and degenerate sequences thereof; substantially pure protease-activated  
10 receptor 3 polypeptides encoded thereby; as well as amino acid sequences substantially identical to the amino acid sequences SEQ ID NO:3 and SEQ ID NO:6 from mouse and human, respectively. The invention further comprises fragments of the PAR3 receptor which are activated by  
15 thrombin. Such fragments may have the same amino acid sequence as SEQ ID NOs:3 and 6 or be at least 80% identical to the amino acid sequences SEQ ID NO:3 and SEQ ID NO:6.

          In various embodiments, the DNA, receptor or  
20 receptor fragment is derived from a vertebrate animal, preferably, human or mouse. However, the gene can be chemically synthesized.

          An object of the invention is to provide a nucleotide sequence encoding a novel receptor.

25           Another object is to provide a cell line genetically engineered to express the nucleotide sequence.

          Another object is to provide a method whereby a compound or library of compounds can be assayed as  
30 thrombin agonists or antagonists for their ability to activate or block the receptor expressed by the nucleotide sequence.

          An advantage of the present invention is that a

- 4 -

antagonists which may not be identifiable via PAR1 or PAR2 receptors.

A feature of the invention is that it makes it possible to obtain additional information regarding  
5 thrombin activation and the sequence of biochemical events initiated by such.

These and other objects, advantages and features of the present invention will become apparent to those skilled in the art upon reading the disclosure.

10

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is the complete nucleotide and amino acid sequences (SEQ ID NO:1 and SEQ ID NO:3, respectively) of the mouse protease-activated receptor 3 gene coding region cDNA. The deduced amino acid sequence of the  
15 receptor is provided below the nucleotide sequence and contains 369 amino acids. The deduced amino acid sequence begins at nucleotides 51-53 (ATG = Met) and ends at nucleotides 1158-1160 (TAG = stop).

Fig. 2 is the genomic sequence (containing exon 2)  
20 of the mouse protease-activated receptor 3 (SEQ ID NO:2).

Fig. 3 is the nucleotide and deduced amino acid sequences (SEQ ID NO:4 and SEQ ID NO:6, respectively) of the human protease-activated gene coding region cDNA. The deduced amino acid sequence is provided below the  
25 nucleotide sequence and contains 374 amino acids. The coding region of the cDNA sequence begins at nucleotides 58-60 (ATG = Met) and ends at nucleotides 1180-1182 (TAG = stop).

Fig. 4 is the genomic sequence (containing exon 2)  
30 of the human protease-activated receptor 3 (SEQ ID NO:5).

Fig. 5A shows the alignment of the deduced amino acid sequences (SEQ ID NO:3, 6, 7, 8, 9) of the mouse PAR3, human PAR3, human PAR1, and human PAR2. To

- 5 -

indicate homology, gaps (represented by blank spaces) have been introduced into the five sequences.

Transmembrane domains are overlined (TM1-7). Fig. 5B shows the alignment of the hirudin-like portion of human  
5 PAR1, PAR2, and PAR3 amino acid sequences.

Fig. 6 is a bar graph showing cell surface binding of M1 monoclonal antibody to M1 epitope on Cos 7 cells expressing hPAR3 or hPAR3 T39P in the presence and absence of  $\alpha$ -thrombin.

10 Fig. 7 is a bar graph of hPAR3 signaling in Cos 7 cells in the presence and absence of G $\alpha$ 16 and the presence and absence of  $\alpha$ -thrombin. Signaling is measured by phosphoinositide hydrolysis.

Fig. 8 is a graph of phosphoinositide hydrolysis  
15 in response to PAR3 signaling as a function of increasing  $\alpha$ -thrombin concentration, and in the presence and absence of G $\alpha$ 16 protein.

Fig. 9 is a graph of phosphoinositide hydrolysis in response to PAR3 signaling as a function of increasing  
20  $\gamma$ -thrombin concentration, and in the presence and absence of G $\alpha$ 16 protein.

Fig. 10 is a graph comparing the specificity of PAR1 and PAR3 for thrombin.

#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

25 Before the present protease-activated receptor assays and methods of using such are described, it is to be understood that this invention is not limited to the particular DNA sequences, materials, methods, or processes described as such may, of course, vary. It is  
30 also to be understood that the terminology used herein is for the purpose of describing particular embodiments

- 6 -

of the present invention will be limited only by the appended claims.

It must be noted that as used in this specification and the appended claims, the singular forms  
5 "a", "and," and "the" include plural referents unless the contexts clearly dictates otherwise. Thus, for example, reference to "a DNA sequence" includes mixtures and large numbers of such sequences, reference to "an assay" includes assays of the same general type, and reference  
10 to "the method" includes one or more methods or steps of the type described herein.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be  
15 construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

Unless defined otherwise, all technical and scientific terms herein have the same meaning as commonly  
20 understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials, similar or equivalent to those described herein, can be used in the practice or testing of the present invention, the preferred methods and materials  
25 are described herein. All publications cited herein are incorporated herein by reference for the purpose of disclosing and describing specific aspects of the invention for which the publication is cited in connection with.

30

## DEFINITIONS

By "protease-activated receptor 3", "PAR3", "PAR3 receptor" and the like, is meant all or part of a vertebrate cell surface protein which is specifically



- 7 -

activated by thrombin or a thrombin agonist thereby activating PAR3-mediated signalling events (e.g., phosphoinositide hydrolysis,  $\text{Ca}^{2+}$  efflux, platelet aggregation). The polypeptide is characterized as having  
5 the ligand activating properties (including the agonist activating and antagonist inhibiting properties) and tissue distribution described herein. Specifically, PAR3 receptors are expressed by the DNA sequences of SEQ ID NOs:2, 4, and 5.

10 By a "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation).

By "substantially pure" is meant that the protease-activated receptor 3 polypeptide provided by the  
15 invention is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, PAR3  
20 polypeptide. A substantially pure PAR3 polypeptide may be obtained, for example, by extraction from a natural source, by expression of a recombinant nucleic acid encoding a PAR3 polypeptide, or by chemically synthesizing the protein. Purity can be measured by any  
25 appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis. The protein is substantially pure if it can be isolated to a band in a gel.

By a "substantially identical" amino acid sequence  
30 is meant an amino acid sequence which differs only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same class (e.g., valine for leucine, arginine for lysine,

- 8 -

positions of the amino acid sequence which do not destroy the biological activity of the receptor. Such equivalent receptors can be isolated by extraction from the tissues or cells of any animal which naturally produce such a  
5 receptor or which can be induced to do so, using the methods described below, or their equivalent; or can be isolated by chemical synthesis; or can be isolated by standard techniques of recombinant DNA technology, e.g., by isolation of cDNA or genomic DNA encoding such a  
10 receptor. Substantially identical receptors have the same biological function, e.g. are activated by the same compound.

By "derived from" is meant encoded by the genome of that organism and present on the surface of a subset  
15 of that organism's cells.

By "isolated DNA" is meant DNA that is not in its native environment in terms of not being immediately contiguous with (i.e., covalently linked to) the complete coding sequences with which it is immediately contiguous  
20 (i.e., one at the 5' end and one at the 3' end) in the naturally-occurring genome of the organism from which the DNA of the invention is derived. The term therefore includes, for example, recombinant DNA which is incorporated into a vector; into an autonomously  
25 replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes any  
30 recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

"Isolated DNA" can mean the DNA is in vectors which are preferably capable of directing expression of the protein encoded by the DNA in a vector-containing  
35 cell and further includes cells containing such vectors

- 9 -

(preferably eukaryotic cells, e.g., CHO cells (ATCC; Cat. No. CCL 61 or COS-7 cells (ATCC; Cat. No. CRL 1651; and the *Xenopus* oocytes of the type described in the above cited reference Vu, T.-K.H. et al. (1991) Cell 64:1057-  
5 1068). Preferably, such cells are stably transfected with such isolated DNA.

By "transformed cell" and "transfected cell", "genetically engineered cell", and the like, is meant a cell into which (or into an ancestor of which) has been  
10 introduced, by means of genetic engineering, a DNA molecule encoding a PAR3 (or DNA encoding a biologically active fragment or analog, thereof). Such a DNA molecule is "positioned for expression" meaning that the DNA molecule is positioned adjacent to a DNA sequence which  
15 directs transcription and translation of the sequence (i.e., facilitates the production of the PAR3 protein, or fragment or analog, thereof).

By "specifically activates", as used herein, is meant an agent, such as thrombin, a thrombin analog, a  
20 PAR3 agonist or other chemical agent including polypeptides such as an antibody, which activates protease-activated receptor 3, receptor polypeptide or a fragment or analog thereof to initiate PAR3-mediated biological events as described herein, but which does not  
25 substantially bind other molecules in a sample, e.g., a biological sample, which naturally includes a protease-activated receptor 3 polypeptide.

By "specifically inhibits", as used herein, is meant an agent, such as a thrombin analog, a PAR3  
30 antagonist or other chemical agent including polypeptides such as an antibody, which inhibits activation of protease-activated receptor 3, receptor polypeptide or a fragment or analog thereof, such as by inhibiting

- 10 -

inhibits the biological activity *in vivo* or *in vitro* of the protein to which it binds.

By "biological activity" is meant the ability of the protease-activated receptor 3 to bind thrombin or a PAR3 agonist and signal the appropriate cascade of biological events (e.g., phosphoinositide hydrolysis,  $\text{Ca}^{2+}$  efflux, and platelet aggregation, and the like, as described herein.

By "substantial increase" is meant an increase in activity or other measurable phenotypic characteristic that is at least approximately a 2-fold increase over control level (where control assays are performed in the absence of activator), preferably at least approximately a 5-fold increase, more preferably at least approximately a 10-fold increase in activity over a control assay.

By "substantial decrease" or "substantial reduction" is meant a decrease or reduction in activity or other measurable phenotypic characteristic that is approximately 80% or the control level, preferably reduced to approximately 50% of the control level, or more preferably reduced to approximately 10% or less of the control level.

The terms "screening method" and "assay method" are used to describe a method of screening a candidate compound for its ability to act as an agonist of a PAR3 ligand. The method involves: a) contacting a candidate agonist compound with a recombinant protease-activated receptor 3 (or PAR3 agonist-binding fragment or analog); b) measuring activation of the receptor, the receptor polypeptide or the receptor fragment or analog; and c) identifying agonist compounds as those which interact with the recombinant receptor and trigger PAR3 activation. Interaction may be cleavage of the receptor to unmask an intramolecular receptor activating peptide or by mimicking the intramolecular receptor-activating

- 11 -

peptide. A tethered ligand may be more difficult to block than a free agonist. Thus, blocking thrombin is the acid test for an agonist which will block responses by other thrombin substrates.

5 By an "agonist" is meant a molecule which mimics a particular activity, in this case, interacting with a PAR3 ligand in a manner which activates thereby triggering the biological events which normally result from the interaction (e.g., phosphoinositide hydrolysis,  
10  $\text{Ca}^{2+}$  efflux, and platelet aggregation). Preferably, an agonist initiates a substantial increase in receptor activity relative to control assays in the absence of activator or candidate agonist. An agonist may possess the same, less, or greater activity than a naturally-  
15 occurring PAR3 ligand.

The terms "antagonist assay", "antagonist screening" and the like, refer to a method of screening a candidate compound for its ability to antagonize interaction between a naturally-occurring activating  
20 ligand or an agonist and the PAR3. The method involves:  
a) contacting a candidate antagonist compound with a first compound which includes a recombinant PAR3 (or agonist-binding fragment or analog) on the one hand and with a second compound which includes thrombin or a PAR3  
25 agonist on the other hand; b) determining whether the first and second compounds interact or are prevented from interaction by the candidate compound; and c) identifying antagonistic compounds as those which interfere with the interaction of the first compound (PAR3 receptor) to the  
30 second compound (PAR3 agonist) and which thereby substantially reduce thrombin or PAR3 agonist-activated biological events (e.g., phosphoinositide hydrolysis,  $\text{Ca}^{2+}$  efflux, and platelet aggregation).

- 12 -

by inhibiting a particular activity such as the ability of thrombin, for example, to interact with a protease-activated receptor 3 thereby triggering the biological events resulting from such an interaction (e.g.,  
5 phosphoinositide hydrolysis,  $\text{Ca}^{2+}$  efflux, and platelet secretion, or platelet aggregation). An antagonist may bind to and thereby block activation of a PAR3 receptor.

The terms "treatment", "treating", "treat" and the like are used herein to generally mean obtaining a  
10 desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the  
15 disease. "Treatment" as used herein covers any treatment of a disease in a mammal, particular a human, and includes:

- (a) preventing the disease or symptom from occurring in a subject which may be predisposed to the  
20 disease or symptom but has not yet been diagnosed as having it;
- (b) inhibiting the disease symptom, i.e., arresting its development; or
- (c) relieving the disease symptom, i.e., causing  
25 regression of the disease.

#### PREFERRED EMBODIMENTS

In preferred embodiments of both screening methods, the recombinant PAR3 is stably expressed by a vertebrate cell which normally presents substantially no  
30 PAR3 on its surface (i.e., a cell which does not exhibit any significant thrombin-mediated phosphoinositide hydrolysis or  $\text{Ca}^{2+}$  efflux in the presence of a PAR activator); the vertebrate cell is a mammalian cell, is a

- 13 -

Rat 1 cell, or a COS 7 cell; and the candidate antagonist or candidate agonist is a thrombin analog, PAR3 peptide fragment or analog or other chemical agent including a polypeptide such as an antibody.

5       The receptor proteins of the invention are likely involved in the activation of vertebrate platelet, leukocyte, and mesenchymal cells in response to wounding, as well as mediating signalling in embryonic development. Such proteins and in particular PAR3 antagonists are  
10 useful therapeutics for the treatment of such conditions as thrombosis, atherosclerosis, restenosis, and inflammation associated with normal wound healing and a variety of diseases including atherosclerosis, restenosis, pulmonary inflammation (ARDS) and  
15 glomerulosclerosis. Preferred therapeutics include  
1) agonists, e.g., thrombin analogs, PAR3 peptide fragments or analogs thereof, or other compounds which mimic the action of thrombin upon interaction with the protease-activated receptor 3 or mimic the action of an  
20 intramolecular receptor activating peptide; and  
2) antagonists, e.g., thrombin analogs, antibodies, or other compounds, which block thrombin or protease-activated receptor 3 function by interfering with the thrombin:receptor interaction or by interfering with the  
25 receptor intramolecular activating peptide. The dosage would be expected to be comparable with current antiinflammatory drugs and should be adjusted based on the age, sex, weight and condition of the patient beginning with small doses and increasing gradually based on  
30 responsiveness and toxicity.

Because the receptor component may now be produced by recombinant techniques and because candidate agonists and antagonists may be screened using transformed,

- 14 -

therapeutics. Isolation of the PAR3 gene (as cDNA or genomic DNA) allows its expression in a cell type which does not normally bear PAR3 on its surface, providing a system for assaying a thrombin:receptor interaction and  
5 receptor activation.

#### EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make  
10 receptor proteins and sequences encoding such proteins and carry out the methodology for finding such DNA sequences and proteins, and are not intended to limit the scope of what is regarded as the invention. Efforts have been made to insure accuracy with respect to numbers used  
15 (e.g., amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts or parts by weight, molecular weight is weight average molecular weight; temperature is in degrees centigrade; and pressure is at or near  
20 atmospheric.

There now follows a description of the cloning and characterization of the cDNA, genomic DNA and the receptor protein of the protease-activated receptor 3 from mouse and human. Expression vectors containing and  
25 capable of expressing the PAR3 DNA, as well as transformed cells containing and expressing the DNA of the invention are also described. Also described are possible PAR3 agonists and antagonists as well as screening assays for receptor agonists and receptor  
30 antagonists.



- 15 -

## EXAMPLE 1

## Isolation of the Mouse Protease-Activated Receptor 3

Rat platelets were used as a source of RNA in the search for and cloning of PAR3 because rat platelets are more abundant than mouse platelets and, like mouse platelets, they do not respond to PAR1 agonist peptides (Connolly, A. et al. (1996) *Nature* 381: 516-519; and Connolly, T.M. et al (1994) *Thromb Haemost* 72: 627-33).

Total RNA was prepared from rat platelets using Trizol reagent (Gibco BRL). cDNA was then prepared using random hexamer primers and the Superscript reverse transcriptase system (Gibco, BRL). cDNA was then used as template for PCR amplification using a Robocycler Gradient 96<sup>®</sup> (Stratagene) and the primers 5'-

GTITACATGCTI (A/C)AC(C/T)TIGCI (A/C/G/T)TIGC (A/C/G/T)GA-3' (SEQ ID NO:10) and 5'-

GGATAIACIACIGCIA(A/G/T) (A/G) (A/T)AIC(G/T) (A/C/G/T)TC-3' (SEQ ID NO:11) at 5 $\mu$ M in 20 $\mu$ M Tris-HCl (pH 8.4), 50 $\mu$ M KCl, 1.5 $\mu$ M MgCl<sub>2</sub>, 0.2 $\mu$ M dNTP, and 50U/ $\mu$ l Taq polymerase.

Polymerase chain reaction temperature was varied as follows: 94°C for 4 min; 30 cycles of 94°C for 45 sec, 39°C for 60 sec, and 72°C for 90 sec; then 72°C for 7 min. PCR products were subcloned using the TA cloning kit (InVitrogen, San Diego, CA). Rat cDNA clones with inserts of approximately 200 bp were analyzed by nucleic acid sequencing. One sequence predicted a novel G-protein coupled receptor related to PAR1 and PAR2.

This sequence was used to obtain mouse and human cDNA and genomic clones by a combination of PCR and hybridization techniques (see, for example, Sambrook, J. et al. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York). The nucleotide sequences

- 16 -

The rat PCR product was then used to clone the full length mouse cDNA and genomic DNA clones. The nucleotide sequences and deduced amino acid sequence of the mouse PAR3 are shown in Figs. 1 and 2.

5       The human PAR3 cDNA used for the functional studies presented below was cloned from a Lamda gt 10 intestinal cDNA library (Clontech). Features of human PAR3's amino acid sequence are shown in Figs. 5A and 5B by alignment of the deduced amino acid sequence of PAR3  
10 with those of PAR1 and PAR2. Predicted transmembrane (TM) domains are overlined and predicted Asn-linked glycosylation sites in PAR3 are underlined in the figure. The amino terminal exodomains are compared in Fig. 5b, including the cleavage site (^), the tethered ligand  
15 domains of PAR1 and PAR2, and the predicted tethered ligand domain of PAR3 (underlined). Also underlined is PAR3's hirudin-like domain (FEEFP). The similar FEEIP and YEPFW sequences in hirudin and PAR1, respectively are known to bind thrombin's fibrinogen-binding exosite.

20       The human PAR3 cDNA contained an open reading frame encoding a 374 amino acid putative G protein-coupled receptor (Fig. 3). BLAST search of the Genbank and EST databases revealed this protein to be novel with 28% and 30% amino acid sequence identity to human PAR1  
25 and PAR2 (Fig. 5a, Table I). Its amino terminal exodomain revealed a possible thrombin cleavage site and a striking hirudin-like sequence (Fig. 5b). Like the carboxyl tail of hirudin itself, PAR1's hirudin-like sequence is known to dock with thrombin's fibrinogen  
30 binding exosite, an interaction important for efficient PAR1 cleavage by thrombin (Vu, T.-K.H. et al. (1991) Nature 353:674-677; Liu, L. et al. (1991) J. Biol. Chem 266:16977-16980; Mathews, I.I. et al. (1994) Biochem 33 3266-79; Ishii, K. (1995) J. Biol. Chem 270:16435-16440,  
35 which references are herein incorporated by reference in

- 17 -

their entirety). These observations strongly suggested that this new receptor was a novel thrombin receptor.

A comparison of PAR deduced amino acid sequences from human, mouse, and *Xenopus* is provided in Table I below. The % identity of the total sequence as well as the % identity of the transmembrane regions are shown.

TABLE I

PAR SEQUENCE	% AMINO ACID IDENTITY	
	TOTAL	TM1-7
hPAR3 vs hPAR1	28	37
hPAR3 vs hPAR2	30	38
hPAR1 vs hPAR2	28	42
hPAR3 vs xPAR1	29	38
hPAR1 vs xPAR1	52	63
hPAR3 vs mPAR3	67	74
hPAR1 vs mPAR1	77	81
hPAR2 vs mPAR2	78	85

h = human

m = mouse

x = *Xenopus laevis*

## EXAMPLE 2

## Polypeptide Expression

Polypeptides according to the invention may be produced by transformation of a suitable host cell with all or part of a PAR3 encoding cDNA fragment (e.g., the

- 18 -

Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems may be used to provide the recombinant receptor protein. The precise host cell used is not critical to the invention. The receptor may be produced in a prokaryotic host (e.g., *E. coli*) or in a eukaryotic host (e.g., *Saccharomyces cerevisiae* or mammalian cells, e.g., COS-6M, COS-7, NIH/3T3, or Chinese Hamster Ovary cells). Such cells are available from a wide range of sources (e.g., the American Type Culture Collection, Rockville, MD). The method of transfection and the choice of expression vehicle will depend on the host system selected. Transformation and mammalian cell transfection methods are described, e.g., in Ausubel et al. (Current Protocols in Molecular Biology, John Wiley & Sons, New York, (1989)); expression vehicles may be chosen from those provided, e.g., in Cloning Vectors: A Laboratory Manual (Pouwels, P.H. et al., (1985), Supp. 1987).

Particularly preferred expression systems are the *Xenopus* oocyte cells of Vu et al. (Vu et al., Cell (1991) *supra*) and insect cells (SF9-baculovirus) transfected with an expression vector containing and expressing a receptor protein or biologically active fragment thereof. DNA encoding the human or mouse PAR3 or an appropriate receptor fragment or analog (as described above) is inserted into the expression vector in an orientation designed to allow expression. Alternatively, the PAR3 (or biologically active receptor fragment or analog) is expressed by a stably-transfected mammalian cell line. Other preferable host cells which may be used in conjunction with the expression vehicle include NIH/3T3 cells (ATCC Accession No. 1658). The expression may be used in a screening method of the invention (described below) or, if desired, the recombinant receptor protein may be isolated as described below.

- 19 -

A number of vectors suitable for stable transfection of mammalian cells are available to the public, e.g., see Pouwels et al. (supra); methods for constructing such cell lines are also publicly available, e.g., in Ausubel et al. (supra). In one example, cDNA encoding the receptor (or receptor fragment or analog) is cloned into an expression vector which includes the dihydrofolate reductase (DHFR) gene. Integration of the plasmid and, therefore, the PAR3-encoding gene into the host cell chromosome is selected for by inclusion of 0.01-300  $\mu$ M methotrexate in the cell culture medium (as described in Ausubel et al., supra). This dominant selection can be accomplished in most cell types. Recombinant protein expression can be increased by DHFR-mediated amplification of the transfected gene. Methods for selecting cell lines bearing gene amplifications are described in Ausubel et al. (supra); such methods generally involve extended culture in medium containing gradually increasing levels of methotrexate. DHFR-containing expression vectors commonly used for this purpose include pCVSEII-DHFR and pAdD26SV(A) (described in Ausubel et al., supra). Any of the host cells described above or, preferably, a DHFR-deficient CHO cell line (e.g., CHO DHFR cells, ATCC Accession No. CRL 9096) are among the host cells preferred for DHFR selection of a stably-transfected cell line or DHFR-mediated gene amplification.

One particularly preferred stable expression system is a Rat 1 cell (ATCC) stably transfected with a pcDNA1/NEO (InVitrogen, San Diego, CA) expression vector.

Expression of the recombinant receptor (e.g., produced by any of the expression systems described herein) may be assayed by immunological procedures, such

- 20 -

intact recombinant cells (using, e.g., the methods described in Ausubel et al., supra). Recombinant receptor protein is detected using an antibody directed to the receptor. Described below are methods for  
5 producing anti-protease-activated receptor 3 antibodies using, as an immunogen, the intact receptor or a peptide which includes a suitable protease-activate receptor 3 epitope. To detect expression of a PAR3 fragment or analog, the antibody is preferably produced using, as an  
10 immunogen, an epitope included in the fragment or analog.

Once the recombinant PAR3 protein (or fragment or analog, thereof) is expressed, it is isolated, e.g., using immunoaffinity chromatography. In one example, an anti-PAR3 antibody may be attached to a column and used  
15 to isolate intact receptor or receptor fragments or analogs. Lysis and fractionation of receptor-harboring cells prior to affinity chromatography may be performed by standard methods (see, e.g., Ausubel et al., supra). Once isolated, the recombinant protein can, if desired,  
20 be further purified, e.g., by high performance liquid chromatography (see, e.g., Fisher, Laboratory Techniques In Biochemistry And Molecular Biology, eds., Work and Burdon, Elsevier, (1980)).

Receptors of the invention, particularly short  
25 receptor fragments, can also be produced by chemical synthesis (e.g., by the methods described in Solid Phase Peptide Synthesis, (1984) 2nd ed., The Pierce Chemical Co., Rockford, IL).

### EXAMPLE 3

#### 30 Cleavage and Activation Studies of the Recombinant Protease-Activated Receptor 3

PAR3 was demonstrated to be a substrate for thrombin when expressed on the surface of Cos 7 cells (Fig. 6). Human PAR1 or PAR3 cDNAs that were modified to

- 21 -

encode receptors displaying a FLAG epitope (amino acid sequence DYKDDD (SEQ ID NO:12) at a site amino to the thrombin cleavage site were transiently expressed in Cos7 cells. Epitope-tagged PAR1 has been previously described  
5 (Ishii, K. et al. (1993) J. Biol. Chem. 268:9780-9786). The analogous epitope-tagged PAR3 cDNA was constructed so as to encode a new amino terminus with the sequence MDSKGSSQKGSRLLLLLLVVSNLLLCQGVVS/DYKDDDDVE-TF (SEQ ID NO:13) representing the prolactin signal peptide,  
10 putative signal peptidase site (/), FLAG epitope DYKDDDD (SEQ ID NO:12) and junction VE fused to amino acid 17 in PAR 3.

cDNAs were subcloned into the mammalian expression vector pBJ1. For receptor cleavage studies Cos 7 cells  
15 were transfected using DEAE-dextran and thrombin-mediated loss of M1 antibody (Kodak) binding to the FLAG epitope of the cell surface using a procedure described by Ishii et al. (Ishii, K. et al. (1993) *supra*). Over 95% of M1 antibody binding was transfection-dependent in this  
20 system. Cells were incubated for 5 min. at 37°C in the presence (open columns) or absence (closed columns) of 20nM thrombin (Fig. 6). For biochemical identification of the cleavage site, cleavage of soluble PAR3 amino terminal exodomain by thrombin was assayed as follows. A  
25 recombinant PAR3 soluble exodomain was prepared in which the amino terminal exodomain residues 21-94 were sandwiched between a translational start and hexahistidine tag (i.e. MG-[PAR3 21-94]-VEHHHHHH; where VEHHHHHH is SEQ ID NO:18). The recombinant protein was  
30 expressed as a soluble polypeptide in *E. coli*, purified, and analyzed before and after thrombin cleavage as previously described for the analogous region of PAR1 (Ishii, K. (1995) J. Biol. Chem. 270:16435-16440).

- 22 -

analyzed by SDS-PAGE. Even prolonged incubation with a high concentration of thrombin yielded only one detectable cleavage event indicating that only one thrombin cleavage site exists in the PAR3 exodomain.

- 5 Amino acid sequencing of the cleavage products revealed only a single new amino terminus with the sequence TFRG (see Fig. 1b). Thus, thrombin recognizes and cleaves PAR3 in the amino terminal exodomain between amino acids K38 and T39 with high specificity.

#### EXAMPLE 4

10

#### PAR3 Signaling Activity

- The ability of PAR3 to mediate signaling by thrombin was tested. *Xenopus* oocytes were microinjected with cRNA encoding epitope-tagged human PAR3 (hPAR3),
- 15 hPAR3 bearing the T39P cleavage site mutation, or the F40A tethered ligand domain mutation. Thrombin-triggered <sup>45</sup>Ca release was measured as described in Vu et al. (Vu, T.-K. H. et al. (1991) *supra*). Surface expression of wild type and mutant receptors was confirmed by M1
- 20 antibody binding by the method of Ishii, K. et al. (Ishii, K. et al. (1995) J. Biol. Chem. 270:16435-16440; and Ishii, K. et al. (1993) J. Biol. Chem. 268:9780-9786, which references are herein incorporated by reference in their entirety).

- 25 Microinjection of *Xenopus* oocytes with human PAR3 cRNA conferred thrombin-dependent <sup>45</sup>Ca mobilization (Fig. 7) which reflects agonist-triggered phosphoinositide hydrolysis in this system. Mutation of PAR3's thrombin cleavage site ablated thrombin signaling
- 30 and thrombin rendered proteolytically inactive by the active site inhibitor PPACK failed to activate PAR3 even at concentrations as high as 1 $\mu$ M. These data strongly



- 23 -

suggest that cleavage of the K 38 -T 39 peptide bond is necessary for PAR3 activation by thrombin.

The specificity of PAR3 and PAR1 signaling was also examined. Protease-triggered  $^{45}\text{Ca}$  release was  
5 measured in *Xenopus* oocytes expressing human PAR1 or PAR3 stimulated with various concentrations of the arginine/lysine specific serine proteases trypsin, Factor Xa, Factor VIIa, tissue plasminogen activator, or plasmin. Chymotrypsin, elastase, and cathepsin G were  
10 also tested. PAR3 was at least as specific for thrombin as thrombin receptor PAR1 (Fig. 10).

PAR3 signaling in Cos 7 cells was also examined. Cos 7 cells were transfected with human PAR1 or PAR3. Cells were then metabolically labelled with  $^3\text{H}$ -inositol  
15 and phosphoinositide hydrolysis was measured in response to the indicated concentrations of  $\alpha$ -thrombin (Fig. 8) or  $\gamma$ -thrombin (Fig. 9) as described by Ishii, et al. and Nanevycz et al. (Ishii, K. et al. (1993) *supra*; and Nanevycz, T. et al. (1996) J. Biol. Chem. 271:702-706).

20 Co-transfection with  $\alpha 16$ , a G protein  $\alpha$ -subunit expressed in hematopoietic cell lines (Amatruda III, T.T. et al. (1991) J. Biol. Chem. 266:5587-5591) caused a 50-150% increase in the maximal PAR3-mediated response to thrombin in these cells in each of three separate  
25 experiments (Fig. 7).

The  $\text{EC}_{50}$  for thrombin signaling through PAR3 in this system was approximately 0.2 nM, comparable to that seen with PAR1 and well within physiologically achievable thrombin concentrations (Fig 8).  $\gamma$ -thrombin, which is  
30 defective in its anion-binding exosite (Rydel, T.J. et al. (1994) J. Biol. Chem. 269:22000-22006), was two log units less potent than  $\alpha$ -thrombin ( $\text{EC}_{50}$  = 20nM; Fig. 9). Similarly, incubation of  $\alpha$ -thrombin with the fibrinogen

- 24 -

dose response curve two logs (not shown). Alanine substitution at F 48 and E 49 in PAR3's hirudin-like sequence, residues predicted to dock with thrombin's fibrinogen-binding exosite by analogy with hirudin and PAR1 (Fig. 5B) also caused a decrease in thrombin signaling by PAR3. These data strongly suggest that PAR3 interacts with thrombin in a manner similar to PAR1 (Mathews, I. I., et al. (1994) *Biochem.* 33:3266-3279). Specifically, it is likely that PAR3 amino acids 48-52 (FEEFP, SEQ ID NO:14) dock with thrombin's fibrinogen-binding exosite while amino acids 35-38 (LTPK, SEQ ID NO:15) dock with thrombin's active center leading to cleavage of the K 38 - T 39 peptide bond.

Synthetic peptides that mimic the new amino terminus unmasked by receptor proteolysis, the so called "tethered ligand domain", act as agonists for PAR1 and PAR2 (Vu, T. K.-H. et al. (1991) *Cell* 64:1057-1068; Nystedt, S. et al. (1994) *PNAS USA* 91:9208-9212; and USPN 5,256,766, which references are herein incorporated by reference in their entirety).

Peptides homologous to the tethered domain of PAR3 may be tested as potential agonists of PAR3 activity. Two peptides, TFRGAP (SEQ ID NO:16) and TFRGAPPNS (SEQ ID NO:17) were synthesized and tested for their ability to mimic the action of thrombin by causing PAR3 signaling as measured by phosphoinositide hydrolysis. Cos 7 cells expressing human PAR3 were incubated with the peptides at concentrations up to 100 $\mu$ M. Phosphoinositide hydrolysis was not observed to be above control levels indicating that the synthetic peptides caused no detectable signaling by PAR3 under these conditions, whereas an EC<sub>50</sub> of 0.2 nM was determined for  $\alpha$ -thrombin under the same assay conditions. These results demonstrate that monitoring phosphoinositide hydrolysis provides a useful

- 25 -

means for assessing potential agonists for activity on PAR3 signaling for use as potential pharmaceuticals.

The tethered ligand domain of PAR3 was required for PAR3 activation by thrombin. Substitution of Ala for  
5 Phe 40 (the F40A PAR3 mutant), which is analogous to the critical Phe 43 in PAR1's tethered ligand (Scarborough, R.M. et al. (1992) J. Biol. Chem. 267:13146-13149), ablated PAR3 signaling but not PAR3 cleavage by thrombin. The observation that cleavage of the Lys 38-Thr 39  
10 peptide bond is necessary for PAR3 activation suggests that PAR3 is probably activated by the same tethered ligand mechanism utilized by PAR1 and 2.

#### EXAMPLE 5

##### PAR3 Tissue Expression in Mouse and Human

15 In situ hybridization of mouse tissue revealed the presence of PAR3 mRNA in megakaryocytes in mouse spleen.

In the tissues examined (brain, eye, thymus, heart, lung, liver spleen, pancreas, stomach, small intestine, colon, kidneys, bladder, uterus, ovary, testis, skeletal  
20 muscle, peripheral nerve, and skin), megakaryocytes in the spleen were the only cells which displayed clearcut hybridization over background. Control samples in which hybridization was performed with a sense strand probe control were negative for all cells. Northern analysis  
25 of mouse tissues for PAR3 mRNA showed signals in spleen and lung, with low levels seen in brain, heart, and other tissues. Spleen is a hematopoietic organ in mouse, and megakaryocytes are sometimes seen trapped in the pulmonary microvasculature. Thus both Northern and in  
30 situ hybridization data suggest that PAR3 is most abundantly expressed in megakaryocytes in the mouse.

- 26 -

The pharmacology of hPAR3 activation in Cos cells resembles that of mouse platelet activation. Both responses show subnanomolar EC<sub>50</sub>s for activation by  $\alpha$ -thrombin and are thrombin active site- and fibrinogen-binding exosite-dependent. These observations support the concept that the mouse homolog of PAR3 is a thrombin receptor that mediates thrombin responses in mouse platelets. Whether human PAR3 function in human platelets remains to be determined.

10 The *in situ* hybridization studies were performed as follows. Anesthetized adult C57BL/6 mice were perfusion-fixed with 4% paraformaldehyde. Organs to be tested were dissected, trimmed, and immersion-fixed for 4 hours in 4% paraformaldehyde. Processed tissues were  
15 embedded in paraffin, and 5 mm sections were cut. Sense or antisense <sup>35</sup>S-riboprobe was transcribed *in vitro* from mouse PAR2 cDNA subcloned into the EcoR1 site of pBluescript II SK<sup>-</sup> (Stratagene, San Diego, CA). Hybridization, wash, and development conditions were as  
20 reported for mouse PAR1 (Soifer, S.J. et al. (1993) Am. J. Pathol. 144:60-69). To carry out Northern analysis a <sup>32</sup>P-labeled probe for the mouse message was generated by random priming (Prime-It II kit; Stratagene) of PCR-amplified DNA fragments corresponding to mouse cDNA  
25 codons representing transmembrane domains 2 to 3. High stringency hybridizations and washes were performed as per the Clontech protocol for Northern analysis.

Northern analysis of human tissues revealed that PAR3 mRNA is widely distributed with signals noted in  
30 small intestine, bone marrow, heart, pancreas, lung, liver, adrenal, trachea, lymph node, stomach, and peripheral blood leukocytes. The role of PAR3 in these various human tissues awaits definition; the finding of PAR3 in human bone marrow and leukocytes is consistent

- 27 -

with PAR3's playing a role in mediating activation of platelets and other hematopoietic cells by thrombin.

#### EXAMPLE 6

##### Assays for Protease-Activated Receptor 3 Function

5           Useful receptor fragments or analogs of the invention are those which interact with thrombin and are activated to initiate the cascade of events associated with thrombin:receptor interaction. Such an interaction may be detected by an *in vitro* functional assay method  
10 (e.g., the phosphoinositide hydrolysis assay, <sup>45</sup>Ca efflux assay, or platelet aggregation assay described herein). This method includes, as components, thrombin and a recombinant protease-activated receptor 3 (or a suitable fragment or analog) configured to permit thrombin binding  
15 (e.g., those polypeptides described herein). Thrombin may be obtained from Sigma Chemical Co. (St. Louis, MO) or similar supplier.

          Preferably, the protease-activated receptor 3 component is produced by a cell that naturally presents  
20 substantially no receptor on its surface, e.g., by engineering such a cell to contain nucleic acid encoding the receptor component in an appropriate expression system. Suitable cells are, e.g., those discussed above with respect to the production of recombinant receptor,  
25 such as Rat 1 cells or COS-7 cells.

#### EXAMPLE 7

##### Screening For Protease-Activated Receptor 3 Activator Antagonists and Agonists Antagonists

- 28 -

compound) and the protease-activated receptor 3, thereby preventing or reducing the cascade of events that are mediated by that interaction. The elements of the screen are a PAR3 activator (such as thrombin), a candidate  
5 antagonist, and recombinant PAR3 (or a suitable receptor fragment or analog, as outlined above) configured to permit detection of PAR3 activator, antagonist, and PAR3 function. An additional element may be a downstream substrate, such as phosphoinositide, the hydrolysis of  
10 which is used to measure thrombin activity (Ishii, K. et al. (1993) *supra*; and Nanevycz, T. et al. (1996) *supra*).

Inhibition of thrombin-induced platelet aggregation may also be used as a means of monitoring an antagonist of PAR3 receptor activation. Thrombin is  
15 incubated with the candidate inhibitory compound (such as a peptide) for 5 minutes, then the mixture is added to washed platelets and platelet activation is followed as platelet ATP secretion by lumiaggregometry (see, for example, Connolly, A.J. et al. *Nature* 381:516-519 (1996);  
20 and USPN 5,256,766). Alternately, platelets are incubated with a candidate PAR 3 antagonist for 5 minutes. Thereafter the response to thrombin is measured.

Inclusion of potential antagonists in the  
25 screening assay along with thrombin allows for the screening and identification of authentic receptor antagonists as those which decrease thrombin-mediated events, such as platelet aggregation.

Appropriate candidate thrombin antagonists include  
30 PAR3 fragments, particularly, fragments of the protein predicted to be extracellular and therefore likely to bind thrombin or the tethered ligand; such fragments would preferably include five or more amino acids.

- 29 -

Candidate PAR 3 antagonists include thrombin analogs as well as other peptide and non-peptide compounds and anti-PAR3 antibodies.

#### AGONISTS

5           Another aspect of the invention features screening for compounds that act as PAR3 ligand agonists. Activation of the PAR3 with thrombin or an agonist leads to a cascade of events (such as phosphoinositide hydrolysis,  $\text{Ca}^{2+}$  efflux, and platelet aggregation),  
10 providing a convenient means for measuring thrombin or other agonist activity.

          The agonist screening assay of the invention utilizes recombinant cells expressing recombinant PAR3 (or a suitable receptor fragment or analog, as outlined  
15 herein) configured to permit detection of PAR3 function. Alternatively, a cell such as a leukocyte, a platelet, or a mesenchymal cell that naturally expresses PAR3 may be used. Other elements of the screen include a detectable downstream substrate of the PAR3 activation, such as  
20 radiolabelled phosphoinositide, the hydrolysis of which to a detectable product indicates PAR3 activation by the candidate agonist.

$^{45}\text{Ca}$  efflux from a cell expressing PAR3 may be used as a means of measuring receptor activation by  
25 candidate agonists (Williams, J.A. et al., (1988) PNAS USA 85:4939-4943; Vu, T.-K. H., et al. (1991) Cell 64:1057-1068; and USPN 5,256,766, which references are herein incorporated by reference in their entirety).  
 $^{45}\text{Ca}$  release by oocytes expressing cRNA encoding PAR3 are  
30 assessed as follows. Briefly, intracellular calcium pools are labeled by incubating groups of 30 oocytes in

- 30 -

The labeled oocytes are washed, then incubated in MBSH II without antibiotics for 90 minutes. Groups of 5 oocytes are selected and placed in individual wells in a 24-well tissue culture plate (Falcon 3047) containing 0.5 ml/well  
5 MBSH II without antibiotics. This medium is removed and replaced with fresh medium every 10 minutes, the harvested medium is analyzed by scintillation counting to determine  $^{45}\text{Ca}$  released by the oocytes during each 10-minute incubation. The 10-minute incubations are  
10 continued until a stable baseline of  $^{45}\text{Ca}$  release per unit time is achieved. Two additional 10-minute collections are obtained, then test medium including agonist is added and agonist-induced  $^{45}\text{Ca}$  release determined.

A voltage clamp assay provides an alternative  
15 method of monitoring agonist activity. Agonist-induced inward chloride currents are measured in voltage-clamped oocytes expressing thrombin receptor encoding cRNA essentially as previously described (Julius, D. et al. Science (1988) 241:558-563, herein incorporated by  
20 reference in its entirety) except that the single electrode voltage-clamp technique is employed.

Platelet aggregation may also be used as a means of monitoring PAR3 receptor activation (see, for example, Connolly, A.J. et al. Nature 381:516-519 (1996). In  
25 particular, mouse platelets may utilize only PAR 3 for thrombin signaling. Human platelets may use both PAR 1 and PAR 3. Thus both would be useful in deleting against function at PAR 3.



- 31 -

An agonist useful in the invention is one which imitates the normal thrombin-mediated signal transduction pathway leading, e.g., to an increase in phosphoinositide hydrolysis. Appropriate candidate agonists include  
5 thrombin analogs or PAR3 tethered ligand domains or other agents which mimic the action of thrombin or the PAR 3 tethered ligand domain. Agonists would be useful for aiding discovery of antagonists.

## EXAMPLE 8

## 10 Anti-Protease-Activated Receptor 3 Antibodies

Protease-activated receptor 3 (or immunogenic receptor fragments or analogs) may be used to raise antibodies useful in the invention. Receptor fragments preferred for the production of antibodies are those  
15 fragments deduced or shown experimentally to be extracellular.

Antibodies directed to PAR3 peptides are produced as follows. Peptides corresponding to all or part of the PAR3 protein are produced using a peptide synthesizer by  
20 standard techniques. The peptides are coupled to KLH with m-maleimide benzoic acid N-hydroxysuccinimide ester. The KLH-peptide is mixed with Freund's adjuvant and injected into animals, e.g. guinea pigs or goats, to produce polyclonal antibodies. Monoclonal antibodies may  
25 be prepared using the PAR3 polypeptides described above and standard hybridoma technology (see, e.g., Kohler et al., Nature (1975) 256:495, 1975; Kohler et al., Eur. J. Immunol. (1976) 6:292; Kohler et al., Eur. J. Immunol. (1976) 6:511; Hammerling et al., in Monoclonal Antibodies and T Cell Hybridomas, Elsevier, NY, (1981); and Ausubel  
30 et al., supra). Antibodies are purified by peptide

- 32 -

Once produced, antibodies are tested for their ability to bind PAR3 by specific binding to the surface of PAR3-transfected cells by Western blot or immunoprecipitation analysis (such as by the methods  
5 described in Ausubel et al., supra).

Antibodies which specifically recognize PAR3 are considered to be likely candidates for useful antagonists; such candidates are further tested for their ability to specifically interfere with the interaction  
10 between thrombin and PAR3 (using the functional antagonist assays described herein). Antibodies which antagonize thrombin:PAR3 binding or PAR3 function are considered to be useful antagonists in the invention.

## EXAMPLE 9

15

## THERAPY

Particularly suitable therapeutics for the treatment of wound healing, thrombosis, atherosclerosis, restenosis, inflammation, and other thrombin-mediated signalling disorders are the agonists and antagonists  
20 described above formulated in an appropriate buffer such as physiological saline. Where it is particularly desirable to mimic a receptor fragment conformation at the membrane interface, the fragment may include a sufficient number of adjacent transmembrane residues. In  
25 this case, the fragment may be associated with an appropriate lipid fraction (e.g., in lipid vesicles or attached to fragments obtained by disrupting a cell membrane). Alternatively, anti-PAR3 antibodies produced as described above may be used as a therapeutic. Again,  
30 the antibodies would be administered in a pharmaceutically-acceptable buffer (e.g., physiological saline). If appropriate, the antibody preparation may be combined with a suitable adjuvant.

- 33 -

Antibodies to PAR 3 are useful antagonists which can be formulated as indicated above. Other therapeutically useful antagonists are peptides derived from PAR3 that bind to and block thrombin and include  
5 formulation comprising a pharmaceutically acceptable carrier and one or more of the following:

- (1) the isolated sequence  
LPIKTFRGAPPNSFEEFPFSALE;
- (2) uncleavable thrombin inhibitor  
10 LPIKPFRGAPPNSFEEFPFSALE where the PAR 3 cleavage site P1' is mutated to block cleavage;
- (3) uncleavable thrombin inhibitor LPI  
(hR)TFRGAPPNSFEEFPFSALE where the PAR 3  
15 cleavage site P1 is mutated to block cleavage;  
hR is beta-homoarginine (the extra methylene group is in the main chain);
- (4) uncleavable thrombin inhibitor  
20 (dF)PRPFRGAPPNSFEEFPFSALE where the good active site binding sequence dFPR is substituted for LPIK; dF is D-Phenylalanine;
- (5) any of (1)-(4) above where all or part of the sequence TFRGAPPNS is replaced with spacer  
25 sequences such as GGG;
- (6) variations and combinations of (1)-(5) which act as antagonists.

The therapeutic preparation is administered in accordance with the condition to be treated. Ordinarily,  
30 it will be administered intravenously, at a dosage, of a duration, and with the appropriate timing to elicit the desired response. Appropriate timing refers to, for

- 34 -

which administration of therapeutic preparation elicits the desired response. Alternatively, it may be convenient to administer the therapeutic orally, nasally, or topically, e.g., as a liquid or a spray. The dosages  
5 are determined to be an amount of the therapeutic agent delivered to an animal that substantially reduces or alleviates disease symptoms. Treatment may be repeated as necessary for substantial reduction or alleviation of disease symptoms.

10 PAR3 activator agonists can be used for the treatment of bleeding. Antagonists may be useful in controlling the formation of clots that cause heart attack and stroke, mediating inflammation and the proliferative responses to injury in normal wound healing  
15 and a variety of diseases including atherosclerosis, restenosis, pulmonary inflammations (ARDS), glomerulosclerosis, etc.

The methods of the invention may be used to screen therapeutic receptor activator agonists and antagonists  
20 for their effectiveness in altering thrombin-mediated biological events, such as phosphoinositide hydrolysis or other cell signalling events by the assays described above. Where a non-human mammal is treated or where a therapeutic for a non-human animal is screened, the PAR3  
25 or receptor fragment or analog or the antibody employed is preferably specific for that species.

#### OTHER EMBODIMENTS

Polypeptides according to the invention include any protease-activated receptors (as described herein).  
30 Such receptors may be derived from any source, but are preferably derived from a vertebrate animal, e.g., a human or mouse. These polypeptides are used, e.g., to

- 35 -

screen for antagonists which disrupt, or agonists which mimic, a thrombin:receptor interaction.

Polypeptides of the invention also include any analog or fragment of a PAR3 capable of interacting with thrombin. Such analogs and fragments may also be used to screen for PAR3 ligand antagonists or agonists. In addition, that subset of receptor fragments or analogs which bind thrombin and are, preferably, soluble (or insoluble and formulated in a lipid vesicle) may be used as antagonists to reduce the *in vivo* concentration of endogenous thrombin, either circulating concentration or local concentration. The efficacy of a receptor analog or fragment is dependent upon its ability to interact with thrombin; such an interaction may be readily assayed using PAR3 functional assays (e.g., those described herein).

Specific receptor analogs of interest include full-length or partial receptor proteins including an amino acid sequence which differs only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same class (e.g., valine for leucine, arginine for lysine, etc.) or by one or more non-conservative amino acid substitutions, deletions, or insertions located at positions of the amino acid sequence which do not destroy the receptors' ability to signal thrombin-mediated events (e.g., as assayed above).

Specific receptor fragments of interest include any portion of the PAR3 which is capable of interacting with thrombin, for example, all or part of the extracellular domains predicted from the deduced amino acid sequence. Such fragments may be useful as antagonists (as described above), and are also useful as

- 36 -

interaction between the receptor and thrombin). The sequence of figure 5B is most likely to bind thrombin. Modification of the (K38/T39) cleavage site for example, substitution of proline for T39 will render peptides  
5 mimicking this site uncleavable. Such peptides will bind thrombin with high affinity.

Extracellular regions of novel protease-activated receptors may be identified by comparison with related proteins of similar structure (e.g., other members of the  
10 G-protein-coupled receptor family); useful regions are those exhibiting homology to the extracellular domains of well-characterized members of the family.

Alternatively, from the primary amino acid sequence, the secondary protein structure and, therefore,  
15 the extracellular domain regions may be deduced semi-empirically using a hydrophobicity/hydrophilicity calculation such as the Chou-Fasman method (see, e.g., Chou and Fasman, Ann. Rev. Biochem. (1978) 47:251). Hydrophilic domains, particularly ones surrounded by  
20 hydrophobic stretches (e.g., transmembrane domains) present themselves as strong candidates for extracellular domains. Finally, extracellular domains may be identified experimentally using standard enzymatic digest analysis, e.g., tryptic digest analysis.

25 Candidate fragments (e.g., any extracellular fragment) are tested for interaction with thrombin by the assays described herein (e.g., the assay described above). Such fragments are also tested for their ability to antagonize the interaction between thrombin and its  
30 endogenous receptor, such as PAR3, using the assays described herein. Analogs of useful receptor fragments (as described above) may also be produced and tested for efficacy as screening components or antagonists (using the assays described herein); such analogs are also  
35 considered to be useful in the invention.

- 37 -

Identification of the receptor(s) that mediate thrombin signaling provides potential targets for the development of drugs that block thrombin's undesirable actions or mimic its desirable activities. Thrombin  
5 receptor antagonists may be used for inhibition of platelet-dependent thrombosis in the setting of unstable angina and myocardial infarction or for blocking thrombin's proinflammatory actions on endothelial cells in the setting of vascular injury. Thrombin receptor  
10 agonists may be used to promote hemostasis and fibroblast proliferation at wound sites.

Unmasked tethered ligand domain peptides may provide lead structures for the development of PAR3 agonists or antagonists.

15 The instant invention is shown and described herein in what is considered to be the most practical, and preferred embodiments. It is recognized, however, that departures may be made therefrom, which are within the scope of the invention, and that obvious  
20 modifications will occur to one skilled in the art upon reading this disclosure.

- 38 -

## SEQUENCE LISTING

## (1) GENERAL INFORMATION

(i) APPLICANT: The Regents of the University of  
California5 (ii) TITLE OF THE INVENTION: Protease Activated  
Uses Thereof

Receptor 3 and

(iii) NUMBER OF SEQUENCES: 23

## (iv) CORRESPONDENCE ADDRESS:

10 (A) ADDRESSEE: Fulbright & Jaworski L.L.P.  
(B) STREET: 865 S. Figueroa Street, 29th Floor  
(C) CITY: Los Angeles  
(D) STATE: CA  
(E) COUNTRY: USA  
(F) ZIP: 90017-2571

## 15 (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette  
(B) COMPUTER: IBM Compatible  
(C) OPERATING SYSTEM: DOS  
(D) SOFTWARE: FastSEQ for Windows Version 2.0

## 20 (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

## 25 (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:

## (viii) ATTORNEY/AGENT INFORMATION:

30 (A) NAME: Berliner, Robert  
(B) REGISTRATION NUMBER: 20,121  
(C) REFERENCE/DOCKET NUMBER: 5555-462

## (ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 213-892-9200  
(B) TELEFAX: 213-680-4518  
(C) TELEX:

## 35 (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

40 (A) LENGTH: 1224 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

	TGACTTTGTA	TACTTAACAA	CATCCTGTAG	CCGGGTCTCA	GGACATCAAG	ATGAAAATCC	60
	TTATCTTGGT	TGCAGCTGGG	CTGCTGTTTC	TGCCAGTCAC	TGTTTGCCAA	AGTGGCATAA	120
45	ATGTTTCAGA	CAACTCAGCA	AAGCCAACCT	TAATATTAA	GAGTTTAAAT	GGGGGTCCCC	180
	AAAATACCTT	TGAAGAATTG	CCACTTTCTG	ACATAGAGGG	CTGGACAGGA	GCCACCACAA	240
	CTATAAAAGC	GGAGTGTCCC	GAGGACAGTA	TTTCAACTCT	CCACGTGAAT	AATGCTACCA	300
	TAGGATACCT	GAGAAGTTCC	TTAAGTACCC	AAGTGATACC	TGCCATCTAT	ATCCTGCTGT	360
	TTGTGGTTGG	TGTACCATCC	AACATCGTGA	CCCTGTGGAA	ACTCTCCTTA	AGGACCAAAAT	420
50	CCATCAGTCT	GGTCATCTTT	CACACCAACC	TGGCCATCGC	AGATCTCCTT	TTCTGTGTCA	480
	CACTGCCATT	TAAGATCGCC	TACCATCTCA	ATGGCAACAA	CTGGGTATTT	GGCGAGGTCA	540
	TGTGCCGGAT	CACCACGGTC	GTTTCTACG	GCAACATGTA	CTGCGCTATC	CTGATCCTCA	600
	CTTGATGGG	CATCAACCGC	TACCTGGCCA	CGGCTCACCC	TTTCACATAC	CAGAAGCTGC	660
	CCAAACGCAG	CTTCTCCTTG	CTCATGTGTG	GCATAGTGTG	GGTCATGGTT	TTCTTATACA	720



- 39 -

TGCTGCCCTT TGTCATCCTG AAGCAGGAGT ACCACCTCGT CCACTCAGAG ATCACCACCT 780  
 GCCACGATGT CGTCGACGCG TGCAGTCCC CATCATCCTT CCGATTCTAC TACTTCGTCT 840  
 CCTTAGCATT CTTTGGGTTT CTCATCCCGT TTGTGATCAT CATCTTCTGT TACACGACTC 900  
 TCATCCACAA ACTTAAATCA AAGGATCGGA TATGGCTGGG CTACATCAAG GCCGCTCTCC 960  
 5 TCATCCTTGT GATTTTCACA ATTTGCTTTG CCCCCACCAA CATCATACTC GTAATCCACC 1020  
 ATGCCAACTA CTACTACCAC AATACCGACA GCTTGACTT TATGTATCTT ATTGCTCTGT 1080  
 GCCTGGGAG CCGAATAGC TGCCTAGATC CATTCTTTA CTTTGTCTG TCGAAAGTTG 1140  
 TAGATCAGCT TAATCCTTAG TCGGCAATGG CAAGACCACT TTAGAGACCA AGGAGAGATA 1200  
 TCTGGGAAGA CATACTGCT TGGC 1224

10 (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1124 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCATATGCTA ATATTTCCTT TCAATTACAG GCATAAATGT TTCAGACAAC TCAGCAAAGC 60  
 20 CAACCTTAAC TATTAAGAGT TTTAATGGGG GTCCCCAAA TACCTTTGAA GAATTCNNNN 120  
 NNNTACAAC CTCCATGTGA ATAATGCTAC CATGGGATAC CTGAGAAGTT CCTTAAGTAC 180  
 CAAAGTGATA CCTGCCATCT ACATCCTGGT GTTTGTGATT GGTGTACCAG CGAACATCGT 240  
 GACCTGTGG AAACCTCTCT CAAGGACCAA ATCCATCTGT CTGGTCATCT TTCACACCAA 300  
 CCTGGCCATC GCGGATCTCC TTTCTGTGT CACGCTGCCG TTTAAGATCN NCCTACCATC 360  
 25 TCAATGGCAA CAACTGGGTA TTTGGCGAGG TCATGTGCCG GATCACCACG GTCGTTTTCT 420  
 ACGGCAACAT GACTGCGCT ANNTCCCTGA TCCTCACCTG CATGGGCATC AACCGCTACC 480  
 TGGCCACGGC TCACCTTTC ACATACCAGA AGCTGCCCAA ACGCAGCTTC TCCATGCTCA 540  
 TGTGTGGCAT GGTGTGGGTC ATGGTTTTCT TATACATGCT GCCCTTTGTC ATCCNNNAAG 600  
 CAGGAGTACC ACCTCGTCCA CTCGAGATC ACCACCTGCC ACGATGTCGT CGACGCGTGC 660  
 30 GANTCCCCAT CATCTTCCG ATTCTACTAC TTCGTCTCT TAGCATTCTT TGGGTTCTCT 720  
 ATCCCGTTTG TGATCATCAT CTTCTGTTAC ACGACTCTCA TCCACAACT TAAATCAAAA 780  
 GATCNGATAT GGCTGGGCTA CATCAAGGCC GTCCTCTCA TCCTTGTA TTTACCATC 840  
 TGCTTCCCC CCACCAAGNN NNNNGATATC TGGGAAGACG TACATGCTTG GCTGACTTGT 900  
 GCATGGCACC ATCAGCTCAA TTTTAATTT TTTAATTTA ATTTAATTTA ATTTATGTT 960  
 35 TTTGAGACAG AGCCTCACTG TGTAGCTTG GCTGGCCTGG CTGGTTCTCT ATTTAGACCA 1020  
 GGTTAGCCTT GAACTCACAG AGATCTGCCT GCTTCTGCCT CCAAGTGCT GGGTTCAACC 1080  
 AGGTCTGGCA AGCGCTCCAT TTTTCAGCTC CTCTGCAACA GTGC 1124

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- 40 (A) LENGTH: 407 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Thr Leu Tyr Thr Xaa Gln His Pro Val Ala Gly Ser Gln Asp Ile Lys  
 1 5 10 15  
 Met Lys Ile Leu Ile Leu Val Ala Gly Leu Leu Phe Leu Pro Val  
 20 25 30  
 Thr Val Cys Gln Ser Gly Ile Asn Val Ser Asp Asn Ser Ala Lys Pro  
 35 40 45  
 Thr Leu Thr Ile Lys Ser Phe Asn Gly Gly Pro Gln Asn Thr Phe Glu  
 50 55 60  
 55 Glu Phe Pro Leu Ser Asp Ile Glu Gly Trp Thr Gly Ala Thr Thr Thr  
 65 70 75 80

- 40 -

Pro Ala Ile Tyr Ile Leu Leu Phe Val Val Gly Val Pro Ser Asn Ile  
 115 120 125  
 Val Thr Leu Trp Lys Leu Ser Leu Arg Thr Lys Ser Ile Ser Leu Val  
 130 135 140  
 5 Ile Phe His Thr Asn Leu Ala Ile Ala Asp Leu Leu Phe Cys Val Thr  
 145 150 155 160  
 Leu Pro Phe Lys Ile Ala Tyr His Leu Asn Gly Asn Asn Trp Val Phe  
 165 170 175  
 10 Gly Glu Val Met Cys Arg Ile Thr Thr Val Val Phe Tyr Gly Asn Met  
 180 185 190  
 Tyr Cys Ala Ile Leu Ile Leu Thr Cys Met Gly Ile Asn Arg Tyr Leu  
 195 200 205  
 Ala Thr Ala His Pro Phe Thr Tyr Gln Lys Leu Pro Lys Arg Ser Phe  
 210 215 220  
 15 Ser Leu Leu Met Cys Gly Ile Val Trp Val Met Val Phe Leu Tyr Met  
 225 230 235 240  
 Leu Pro Phe Val Ile Leu Lys Gln Glu Tyr His Leu Val His Ser Glu  
 245 250 255  
 20 Ile Thr Thr Cys His Asp Val Val Asp Ala Cys Glu Ser Pro Ser Ser  
 260 265 270  
 Phe Arg Phe Tyr Tyr Phe Val Ser Leu Ala Phe Phe Gly Phe Leu Ile  
 275 280 285  
 Pro Phe Val Ile Ile Ile Phe Cys Tyr Thr Thr Leu Ile His Lys Leu  
 290 295 300  
 25 Lys Ser Lys Asp Arg Ile Trp Leu Gly Tyr Ile Lys Ala Val Leu Leu  
 305 310 315 320  
 Ile Leu Val Ile Phe Thr Ile Cys Phe Ala Pro Thr Asn Ile Ile Leu  
 325 330 335  
 30 Val Ile His His Ala Asn Tyr Tyr Tyr His Asn Thr Asp Ser Leu Tyr  
 340 345 350  
 Phe Met Tyr Leu Ile Ala Leu Cys Leu Gly Ser Leu Asn Ser Cys Leu  
 355 360 365  
 Asp Pro Phe Leu Tyr Phe Val Met Ser Lys Val Val Asp Gln Leu Asn  
 370 375 380  
 35 Pro Xaa Ser Ala Met Ala Arg Pro Leu Xaa Arg Pro Arg Arg Asp Ile  
 385 390 395 400  
 Trp Glu Asp Ile His Ala Trp  
 405

## (2) INFORMATION FOR SEQ ID NO:4:

40 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1224 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGCTCCATGA TTTTACAGAT TTCATAACGT TTAAGAGACG GGAATCAGGT CATCAAAATG 60  
 AAAGCCCTCA TCTTTGCAGC TGCTGGCCTC CTGCTTCTGT TGCCCACTTT TTGTCAGAGT 120  
 GGCATGGAAA ATGATACAAA CAACTTGCCA AAGCCAACCT TACCCATTAA GACCTTTCTG 180  
 50 GGAGCTCCCC CAAATTCTTT TGAAGAGTTC CCCTTTTCTG CCTTGAAGG CTGGACAGGA 240  
 GCCACGATTA CTGTAAAAAT TAAGTGCCCT GAAGAAAGTG CTTACATCT CCAATGTGAAA 300  
 AATGTACCA TGGGGTACCT GACCAGCTCC TTAAGTACTA AACTGATACC TGCCATCTAC 360  
 CTCCTGGTGT TTGTAGTTGG TGTCCCGGCC AATGCTGTGA CCCTGTGGAT GCTTTTCTTC 420  
 AGGACCCAGAT CCATCTGTAC CACTGTATTG TACACCAACC TGCCATTGCA AGATTTTCTT 480  
 55 TTTTGTGTTA CATTGCCCTT TAAGATAGCT TATCATCTCA ATGGGAACAA CTGGGTATTT 540  
 GGAGAGGTCC TGTGCCGGGC CACCACAGTC ATCTTCTATG GCAACATGTA CTGCTCCATT 600  
 CTGCTCCTTG CCTGCATCAG CATCAACCGC TACCTGGCCA TCGTCCATCC TTTCACCTAC 660  
 CGGGGCGCTGC CCAAGCACAC CTATGCCTTG GTAACATGTG GACTGGTGTG GGCAACAGTT 720  
 TTCTTATATA TGCTGCCATT TTTCATACTG AAGCAGGAAT ATTATCTTGT TCAGCCAGAC 780  
 60 ATCACCACCT GCCATGATGT TCACAACACT TGCGAGTCCT CATCTCCCTT CCAACTCTAT 840  
 TACTTCATCT CCTTGGCATT CTTTGGATTG TTAATTCCAT TTGTGCTTAT CATCTACTGC 900  
 TATGCAGCCA TCATCCGGAC ACTTAATGCA TACGATCATA GATGGTTGTG GTATGTTAAG 960  
 GCGAGTCTCC TCATCCTTGT GATTTTACC ATTTGCTTTG CTCCAAGCAA TATTATCTT 1020  
 ATTATTACAC ATGCTAACTA CTAATAAAT AACACTGATG GCTTATATTT TATATATCTC 1080  
 65 ATAGCTTTGT GCCTGGGTAG TCTTAATAGT TGCTTAGATC CATTCTTTTA TTTTCTCATG 1140

- 41 -

TCAAAAACCA GAAATCACTC CACTGCTTAC CTTACAAAAT AGTGAAATGA TCTTAGAGAA 1200  
CAAGGACAGC CATCACAGAG AACG 1224

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 1102 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: Genomic DNA

## 10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ACAGGCATGG AAAATGATAC AAACAACCTTG GCAAAGCCAA CCTTACCCAT TAAGACCTTT 60  
CGTGGAGCTC CCCCAAATTC TTTTGAAGAG TTCCCTTTT CTGCCTTGA AGGCTGGACA 120  
GGAGCCACGA TTACTGTAAA AATTAAGTGC CCTGAAGAAA GTGCTTCACA TCTCCATGTG 180  
AAAAATGCTA CCATGGGGTA CCTGACCAGC TCCTTAAGTA CTAAACTGAT ACCTGCCATC 240  
15 TACCTCCTGG TGTITGTAGT TGGTGTCCCG GCCAATGCTG TGACCCTGTG GATGCTTTTC 300  
TTCAGGACCA GATCCATCTG TACCACTGTA TTCTACACCA ACCTGGCCAT TGCAGATTTT 360  
CTTTTTTGTG TTACATTGCC CTTTAAGATA GCTTATCATC TCAATGGGAA CAACTGGGTA 420  
TTTGGAGAGG TCCTGTGCCG GGCCACCACA GTCATCTTCT ATGGCAACAT GACTGCTCC 480  
ATTCTGCTCC TTGCTGTCAT CAGCATCAAC CGCTACCTGG CCATCGTCCA TCCTTTCACC 540  
20 TACCGGGGCC TGCCCAAGCA CACCTATGCC TTGGTAACAT GTGGACTGGT GTGGGCAACA 600  
GTTTTCTTAT ATATGCTGCC ATTTTTCATA CTGAAGCAGG AATATTATCT TGTTCAAGCA 660  
GACATCAGCA CCTGCCATGA TGTTCACAAC ACTTGCGAGT CCTCATCTCC CTTCCAACCTC 720  
TATTACTTCA TCTCCTTGGC ATTCTTTGGA TTCTTAATTC CATTTGTGCT TATCATCTAC 780  
TGCTATGCAG CCATCATCCG GACACTTAAT GCATACGATC ATAGATGGTT GTGGTATGTT 840  
25 AAGGCGAGTC TCCTCATCCT TGTGATTTT ACCATTTGCT TTGCTCCAAG CAATATTATT 900  
CTTATTATTC ACCATGCTAA CTACTACTAC AACAACACTG ATGGCTTATA TTTTATATAT 960  
CTCATAGCTT TGTGCTGGG TAGTCTTAAT AGTTGCTTAG ATCCATTCCCT TTATTTTCTC 1020  
ATGTCAAAAA CCAGAAATCA CTCCACTGCT TACCTTACAA AATAGTGAAA TGATCTTAGA 1080  
GAACAAGGAC AGCCATCACA GA 1102

## 30 (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 408 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
35 (D) TOPOLOGY: linear

## (ix) FEATURE:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Cys Ser Met Ile Leu Gln Ile Ser Xaa Arg Leu Arg Asp Gly Thr Gln  
1 5 10 15  
40 Val Ile Lys Met Lys Ala Leu Ile Phe Ala Ala Ala Gly Leu Leu Leu  
20 25 30  
Leu Leu Pro Thr Phe Cys Gln Ser Gly Met Glu Asn Asp Thr Asn Asn  
35 40 45  
Leu Ala Lys Pro Thr Leu Pro Ile Lys Thr Phe Arg Gly Ala Pro Pro  
45 50 55 60  
Asn Ser Phe Glu Glu Phe Pro Phe Ser Ala Leu Glu Gly Trp Thr Gly  
65 70 75 80  
Ala Thr Ile Thr Val Lys Ile Lys Cys Pro Glu Glu Ser Ala Ser His  
85 90 95  
50 Leu His Val Lys Asn Ala Thr Met Gly Tyr Leu Thr Ser Ser Leu Ser  
100 105 110  
Thr Lys Leu Ile Pro Ala Ile Tyr Leu Leu Val Phe Val Val Gly Val  
115 120 125  
Pro Ala Asn Ala Val Thr Leu Trp Met Leu Phe Phe Arg Thr Arg Ser  
55 130 135 140  
Ile Cys Thr Thr Val Phe Tyr Thr Asn Leu Ala Ile Ala Asp Phe Leu  
145 150 155 160

- 42 -

180 185 190  
 Tyr Gly Asn Met Tyr Cys Ser Ile Leu Leu Leu Ala Cys Ile Ser Ile  
 195 200 205  
 5 Asn Arg Tyr Leu Ala Ile Val His Pro Phe Thr Tyr Arg Gly Leu Pro  
 210 215 220  
 Lys His Thr Tyr Ala Leu Val Thr Cys Gly Leu Val Trp Ala Thr Val  
 225 230 235 240  
 Phe Leu Tyr Met Leu Pro Phe Phe Ile Leu Lys Gln Glu Tyr Tyr Leu  
 245 250 255  
 10 Val Gln Pro Asp Ile Thr Thr Cys His Asp Val His Asn Thr Cys Glu  
 260 265 270  
 Ser Ser Ser Pro Phe Gln Leu Tyr Tyr Phe Ile Ser Leu Ala Phe Phe  
 275 280 285  
 Gly Phe Leu Ile Pro Phe Val Leu Ile Ile Tyr Cys Tyr Ala Ala Ile  
 290 295 300  
 15 Ile Arg Thr Leu Asn Ala Tyr Asp His Arg Trp Leu Trp Tyr Val Lys  
 305 310 315 320  
 Ala Ser Leu Leu Ile Leu Val Ile Phe Thr Ile Cys Phe Ala Pro Ser  
 325 330 335  
 20 Asn Ile Ile Leu Ile Ile His His Ala Asn Tyr Tyr Tyr Asn Asn Thr  
 340 345 350  
 Asp Gly Leu Tyr Phe Ile Tyr Leu Ile Ala Leu Cys Leu Gly Ser Leu  
 355 360 365  
 Asn Ser Cys Leu Asp Pro Phe Leu Tyr Phe Leu Met Ser Lys Thr Arg  
 370 375 380  
 25 Asn His Ser Thr Ala Tyr Leu Thr Lys Xaa Xaa Asn Asp Leu Arg Glu  
 385 390 395 400  
 Gln Gly Gln Pro Ser Gln Arg Thr  
 405

30 (2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 425 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

35 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met Gly Pro Arg Arg Leu Leu Leu Val Ala Ala Cys Phe Ser Leu Cys  
 1 5 10 15  
 40 Gly Pro Leu Leu Ser Ala Arg Thr Arg Ala Arg Arg Pro Glu Ser Lys  
 20 25 30  
 Ala Thr Asn Ala Thr Leu Asp Pro Arg Ser Phe Leu Leu Arg Asn Pro  
 35 40 45  
 Asn Asp Lys Tyr Glu Pro Phe Trp Glu Asp Glu Glu Lys Asn Glu Ser  
 50 55 60  
 Gly Leu Thr Glu Tyr Arg Leu Val Ser Ile Asn Lys Ser Ser Pro Leu  
 65 70 75 80  
 Gln Lys Gln Leu Pro Ala Phe Ile Ser Glu Asp Ala Ser Gly Tyr Leu  
 85 90 95  
 50 Thr Ser Ser Trp Leu Thr Leu Phe Val Pro Ser Val Tyr Thr Gly Val  
 100 105 110  
 Phe Val Val Ser Leu Pro Leu Asn Ile Met Ala Ile Val Val Phe Ile  
 115 120 125  
 Leu Lys Met Lys Val Lys Lys Pro Ala Val Val Tyr Met Leu His Leu  
 130 135 140  
 55 Ala Thr Ala Asp Val Leu Phe Val Ser Val Leu Pro Phe Lys Ile Ser  
 145 150 155 160  
 Tyr Tyr Phe Ser Gly Ser Asp Trp Gln Phe Gly Ser Glu Leu Cys Arg  
 165 170 175  
 60 Phe Val Thr Ala Ala Phe Tyr Cys Asn Met Tyr Ala Ser Ile Leu Leu  
 180 185 190  
 Met Thr Val Ile Ser Ile Asp Arg Phe Leu Ala Val Val Tyr Pro Met  
 195 200 205  
 65 Gln Ser Leu Ser Trp Arg Thr Leu Gly Arg Ala Ser Phe Thr Cys Leu  
 210 215 220

- 43 -

Ala Ile Trp Ala Leu Ala Ile Ala Gly Val Val Pro Leu Val Leu Lys  
 225 230 235 240  
 Glu Gln Thr Ile Gln Val Pro Gly Leu Asn Ile Thr Thr Cys His Asp  
 245 250 255  
 5 Val Leu Asn Glu Thr Leu Leu Glu Gly Tyr Tyr Ala Tyr Tyr Phe Ser  
 260 265 270  
 Ala Phe Ser Ala Val Phe Phe Phe Val Pro Leu Ile Ile Ser Thr Val  
 275 280 285  
 Cys Tyr Val Ser Ile Ile Arg Cys Leu Ser Ser Ser Ala Val Ala Asn  
 290 295 300  
 10 Arg Ser Lys Lys Ser Arg Ala Leu Phe Leu Ser Ala Ala Val Phe Cys  
 305 310 315 320  
 Ile Phe Ile Ile Cys Phe Gly Pro Thr Asn Val Leu Leu Ile Ala His  
 325 330 335  
 15 Tyr Ser Phe Leu Ser His Thr Ser Thr Thr Glu Ala Ala Tyr Phe Ala  
 340 345 350  
 Tyr Leu Leu Cys Val Cys Val Ser Ser Ile Ser Ser Cys Ile Asp Pro  
 355 360 365  
 20 Leu Ile Tyr Tyr Tyr Ala Ser Ser Glu Cys Gln Arg Tyr Val Tyr Ser  
 370 375 380  
 Ile Leu Cys Cys Lys Glu Ser Ser Asp Pro Ser Ser Tyr Asn Ser Ser  
 385 390 395 400  
 Gly Gln Leu Met Ala Ser Lys Met Asp Thr Cys Ser Ser Asn Leu Asn  
 405 410 415  
 25 Asn Ser Ile Tyr Lys Lys Leu Leu Thr  
 420 425

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 394 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

35 Met Arg Ser Pro Ser Ala Ala Trp Leu Leu Gly Ala Ala Ile Leu Leu  
 1 5 10 15  
 Ala Ala Ser Leu Ser Cys Ser Gly Thr Ile Gln Gly Thr Asn Arg Ser  
 20 25 30  
 Ser Lys Gly Arg Ser Leu Ile Gly Lys Val Asp Gly Thr Ser His Val  
 35 40 45  
 40 Thr Gly Lys Gly Val Thr Val Glu Thr Val Phe Ser Val Asp Glu Phe  
 50 55 60  
 Ser Ala Ser Val Leu Thr Gly Lys Leu Thr Thr Val Phe Leu Pro Ile  
 65 70 75 80  
 45 Val Tyr Thr Ile Val Phe Val Val Gly Leu Pro Ser Asn Gly Met Ala  
 85 90 95  
 Leu Trp Val Phe Leu Phe Arg Thr Lys Lys Lys His Pro Ala Val Ile  
 100 105 110  
 Tyr Met Ala Asn Leu Ala Leu Ala Asp Leu Leu Ser Val Ile Trp Phe  
 115 120 125  
 50 Pro Leu Lys Ile Ala Tyr His Ile His Gly Asn Asn Trp Ile Tyr Gly  
 130 135 140  
 Glu Ala Leu Cys Asn Val Leu Ile Gly Phe Phe Tyr Gly Asn Met Tyr  
 145 150 155 160  
 55 Cys Ser Ile Leu Phe Met Thr Cys Leu Ser Val Gln Arg Tyr Trp Val  
 165 170 175  
 Ile Val Asn Pro Met Gly His Ser Arg Lys Lys Ala Asn Ile Ala Ile  
 180 185 190  
 Gly Ile Ser Leu Ala Ile Trp Leu Leu Ile Leu Leu Val Thr Ile Pro  
 195 200 205  
 60 Leu Tyr Val Val Lys Gln Thr Ile Phe Ile Pro Ala Leu Asn Ile Thr

- 44 -

245                      250                      255  
 Ser Ala Tyr Val Leu Met Ile Arg Met Leu Arg Ser Ser Ala Met Asp  
                          260                      265                      270  
 5    Glu Asn Ser Glu Lys Lys Arg Lys Arg Ala Ile Lys Leu Ile Val Thr  
                          275                      280                      285  
 Val Leu Ala Met Tyr Leu Ile Cys Phe Thr Pro Ser Asn Leu Leu Leu  
                          290                      295                      300  
 Val Val His Tyr Phe Leu Ile Lys Ser Gln Gly Gln Ser His Val Tyr  
                          305                      310                      315                      320  
 10    Ala Leu Tyr Ile Val Ala Leu Cys Leu Ser Thr Leu Asn Ser Cys Ile  
                          325                      330                      335  
 Asp Pro Phe Val Tyr Tyr Phe Val Ser His Asp Phe Arg Asp His Ala  
                          340                      345                      350  
 Lys Asn Ala Leu Leu Cys Arg Ser Val Arg Thr Val Lys Gln Met Gln  
                          355                      360                      365  
 15    Val Ser Leu Thr Ser Lys Lys His Ser Arg Lys Ser Ser Ser Tyr Ser  
                          370                      375                      380  
 Ser Ser Ser Thr Thr Val Lys Thr Ser Tyr  
                          385                      390

20                      (2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

25                      (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Asp Phe Glu Glu Ile Pro Glu Glu Tyr Leu Gln  
 1                                      5                                      10

30                      (2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

35                      (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: Other

(B) LOCATION: 1...29

40                      (D) OTHER INFORMATION: N=Inosine at residues 3, 12, 18, 21, and 24

(A) NAME/KEY: Other

(B) LOCATION: 22...27

45                      (D) OTHER INFORMATION: N=A or C or G or T at residues 22 and 27

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GTNTACATGC TNMACYTNGC NNTNGCNGA

29

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

50                      (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

55                      (ix) FEATURE:

- 45 -

(A) NAME/KEY: Other  
 (B) LOCATION: 6...21  
 (D) OTHER INFORMATION: N=Inosine at residues 6, 9, 12, 15, and 21

5 (A) NAME/KEY: Other  
 (B) LOCATION: 24  
 (D) OTHER INFORMATION: N=A or C or G or T at residue 24

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

10 GGATANACNA CNGCNADRW A NCKNTC

26

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids  
 (B) TYPE: amino acid  
 15 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Asp Tyr Lys Asp Asp Asp  
 1 5

20 (2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 amino acids  
 (B) TYPE: amino acid  
 25 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Met Asp Ser Lys Gly Ser Ser Gln Lys Gly Ser Arg Leu Leu Leu Leu  
 1 5 10 15  
 30 Leu Val Val Ser Asn Leu Leu Leu Cys Gln Gly Val Val Ser Asp Tyr  
 20 25 30  
 Lys Asp Asp Asp Asp Val Glu  
 35

(2) INFORMATION FOR SEQ ID NO:14:

35 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Phe Glu Glu Phe Pro  
 1 5

(2) INFORMATION FOR SEQ ID NO:15:

45 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4 amino acids

- 46 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Leu Thr Pro Lys  
1

(2) INFORMATION FOR SEQ ID NO:16:

- 5 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 6 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

- 10 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Thr Phe Arg Gly Ala Pro  
1 5

(2) INFORMATION FOR SEQ ID NO:17:

- 15 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 9 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

- 20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Thr Phe Arg Gly Ala Pro Pro Asn Ser  
1 5

(2) INFORMATION FOR SEQ ID NO:18:

- 25 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 23 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein

- 30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Leu Pro Ile Lys Thr Phe Arg Gly Ala Pro Pro Asn Ser Phe Glu Glu  
1 5 10 15  
Phe Pro Phe Ser Ala Leu Glu  
20

- 35 (2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 23 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

40

- (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Leu Pro Ile Lys Pro Phe Arg Gly Ala Pro Pro Asn Ser Phe Glu Glu  
1 5 10 15  
45 Phe Pro Phe Ser Ala Leu Glu  
20

(2) INFORMATION FOR SEQ ID NO:20:



- 47 -

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

5

## (ix) FEATURE:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Leu Pro Ile Xaa Thr Pro Phe Arg Gly Ala Pro Pro Asn Ser Phe Glu  
 1 5 10 15  
 10 Glu Phe Pro Phe Ser Ala Leu Glu  
 20

## (2) INFORMATION FOR SEQ ID NO:21:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

15

## (ix) FEATURE:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

20 Xaa Pro Arg Pro Phe Arg Gly Ala Pro Pro Asn Ser Phe Glu Glu Phe  
 1 5 10 15  
 Pro Phe Ser Ala Leu Glu  
 20

## (2) INFORMATION FOR SEQ ID NO:22:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

25

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Leu Pro Ile Lys  
 1

## (2) INFORMATION FOR SEQ ID NO:23:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

35

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

40

Thr Phe Arg Gly Ala Pro Pro Asn Ser  
 1 5

- 48 -

## CLAIMS

That which is claimed is:

1. Substantially pure DNA encoding a protease-activated receptor 3.

5           2. The DNA of claim 1, wherein the DNA is mammalian.

          3. Substantially pure DNA having the nucleotide sequence selected from the group consisting of Fig. 1 (SEQ ID NO:1), or degenerate variants thereof, and  
10 encoding the amino acid sequence of Fig. 1 (SEQ ID NO:3); Fig. 2 (SEQ ID NO:2), or degenerate variants thereof encoding an amino acid sequence comprising the amino acid sequence of Fig. 1 (SEQ ID NO:3); Fig. 3 (SEQ ID NO:4), or degenerate variants thereof encoding the amino acid  
15 sequence of Fig. 2 (SEQ ID NO:6); and Fig. 4 (SEQ ID NO:5), or degenerate variants thereof encoding an amino acid sequence comprising the amino acid sequence of Fig. 3 (SEQ ID NO:6).

          4. Substantially pure DNA having 50% or greater  
20 sequence identity to the DNA sequence of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:5 and which hybridizes to the DNA sequence SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:5, respectively.

25           5. An isolated protease-activated receptor 3 protein.

- 49 -

6. The substantially pure protein of claim 5 having an amino acid sequence selected from the group consisting of the sequence shown in Fig. 1 (SEQ ID NO:3) and the sequence shown in Fig. 2 (SEQ ID NO:6).

5 7. A substantially pure polypeptide having an amino acid sequence which is at least 80% identical to an amino acid sequence selected from the group consisting of the sequence shown in Fig. 1 (SEQ ID NO:3) and the sequence shown in Fig. 2 (SEQ ID NO:6), wherein

10 a) said polypeptide is activated by thrombin; and  
b) said polypeptide mediates phosphoinositide hydrolysis in a cell expressing said polypeptide on its surface.

8. A substantially pure polypeptide which is a  
15 fragment or analog of a protease-activated receptor 3 comprising a domain capable of activation by thrombin and mediating phosphoinositide hydrolysis.

9. A vector comprising the DNA of claim 1.

10. A cell comprising the vector of claim 9.

20 11. An assay device, comprising:  
a support surface;  
and a cell of claim 10.

12. The assay device of claim 11, wherein the cell is bound to the support surface or present in a  
25 suspension on the support surface.

- 50 -

13. A method of testing a candidate compound for its ability to act as an agonist of a protease-activated receptor 3 ligand, the method comprising:

- a) contacting a candidate compound with a cell  
5 which expresses on its surface a recombinant protease-activated receptor 3 protein or biologically active fragment or analog thereof;
- b) measuring PAR3-mediated response of the cell;  
and
- 10 c) identifying the candidate compound as an agonist wherein the contacting causes a substantial increase in PAR3-mediated response.

14. A method of testing a candidate compound for the ability to act as an antagonist of a protease-  
15 activated receptor 3 ligand, the method comprising:

- a) contacting in the presence of a protease-activated receptor agonist a candidate compound with a cell which expresses on its surface a recombinant protease-activated receptor 3 protein or biologically  
20 active fragment or analog thereof;
- b) measuring PAR3-mediated response of the cell;  
and
- c) identifying the candidate compound as an antagonist wherein the contacting causes a substantial  
25 decrease in PAR3-mediated response relative to PAR3-mediated response in the absence of the candidate antagonist.

15. The method of claim 14, wherein the cell is a mammalian cell which normally presents substantially no  
30 protease-activated receptor 3 on its surface, the PAR3-mediated response measured in intracellular phosphoinositide hydrolysis in the cell.

- 51 -

16. The method of claim 14, further comprising:  
mixing thrombin with platelets and the identified  
candidate compound; and  
observing the effect of the candidate compound on  
5 mediating platelet aggregation.

17. A therapeutic composition, comprising:  
a protease-activated receptor 3 ligand agonist;  
and  
a physiologically-acceptable carrier.

10 18. A therapeutic composition, comprising:  
a protease-activated receptor 3 ligand antagonist;  
and  
a physiologically-acceptable carrier.

- 52 -

19. The composition of claim 18, wherein the antagonist is selected from the group consisting of:

- (1) the isolated sequence  
LPIKTFRGAPPNSFEEFPFSALE;
- 5 (2) uncleavable thrombin inhibitor  
LPIKPFRGAPPNSFEEFPFSALE where the PAR 3  
cleavage site P1' is muted to block cleavage;
- (3) uncleavable thrombin inhibitor LP  
(hR)TFRGAPPNSFEEFPFSALE where the PAR 3  
10 cleavage site P1 is mutated to block  
cleavage;  
hR is beta-homoarginine (the extra methylene  
group is in the main chain);
- (4) uncleavable thrombin inhibitor  
15 (dF)PRPFRGAPPNSFEEFPFSALE where the good  
active site binding sequence dFPR is  
substituted for LPIK; dF is D-Phenylalanine;
- (5) any of (1)-(4) above where all or part of the  
sequence TFRGAPPNS is replaced with spacer  
20 sequences such as GGG;
- (6) variations and combinations of (1)-(5) which  
act as antagonists.

20. A method of treatment, comprising:  
administering to a patient a therapeutically  
25 effective amount of the composition of claim 18.

1/16

## FIG. 1-1

```

      10      20      30      40      50
      *      *      *      *      *
TG ACT TTG TAT ACT TAA CAA CAT CCT GTA GCC GGG TCT CAG GAC ATC AAG
AC TGA AAC ATA TGA ATT GTT GTA GGA CAT CGG CCC AGA GTC CTG TAG TTC
  T  L  Y  T  *  Q  H  P  V  A  G  S  Q  D  I  K>
      60      70      80      90
      *      *      *      *      *
ATG AAA ATC CTT ATC TTG GTT GCA GCT GGG CTG CTG TTT CTG CCA GTC
TAC TTT TAG GAA TAG AAC CAA CGT CGA CCC GAC GAC AAA GAC GGT CAG
  M  K  I  L  I  L  V  A  A  G  L  L  F  L  P  V>
100      110      120      130      140
      *      *      *      *      *
ACT GTT TGC CAA AGT GGC ATA AAT GTT TCA GAC AAC TCA GCA AAG CCA
TGA CAA ACG GTT TCA CCG TAT TTA CAA AGT CTG TTG AGT CGT TTC GGT
  T  V  C  Q  S  G  I  N  V  S  D  N  S  A  K  P>
150      160      170      180      190
      *      *      *      *      *
ACC TTA ACT ATT AAG AGT TTT AAT GGG GGT CCC CAA AAT ACC TTT GAA
TGG AAT TGA TAA TTC TCA AAA TTA CCC CCA GGG GTT TTA TGG AAA CTT
  T  L  T  I  K  S  F  N  G  G  P  Q  N  T  F  E>
200      210      220      230      240
      *      *      *      *      *
GAA TTC CCA CTT TCT GAC ATA GAG GGC TGG ACA GGA GCC ACC ACA ACT
CTT AAG GGT GAA AGA CTG TAT CTC CCG ACC TGT CCT CGG TGG TGT TGA
  E  F  P  L  S  D  I  E  G  W  T  G  A  T  T  T>
250      260      270      280      290
      *      *      *      *      *
ATA AAA GCG GAG TGT CCC GAG GAC AGT ATT TCA ACT CTC CAC GTG AAT
TAT TTT CGC CTC ACA GGG CTC CTG TCA TAA AGT TGA GAG GTG CAC TTA
  I  K  A  E  C  P  E  D  S  I  S  T  L  H  V  N>
300      310      320      330
      *      *      *      *      *
AAT GCT ACC ATA GGA TAC CTG AGA AGT TCC TTA AGT ACC CAA GTG ATA
TTA CGA TGG TAT CCT ATG GAC TCT TCA AGG AAT TCA TGG GTT CAC TAT
  N  A  T  I  G  Y  L  R  S  S  L  S  T  Q  V  I>
340      350      360      370      380
      *      *      *      *      *
CCT GCC ATC TAT ATC CTG CTG TTT GTG GTT GGT GTA CCA TCC AAC ATC
GGA CGG TAG ATA TAG GAC GAC AAA CAC CAA CCA CAT GGT AGG TTG TAG
  P  A  I  Y  I  L  L  F  V  V  G  V  P  S  N  I>
390      400      410      420      430
      *      *      *      *      *

```

2/16

FIG. 1-2

```

      440      450      460      470      480
*   *   *   *   *   *   *   *   *   *
ATC TTT CAC ACC AAC CTG GCC ATC GCA GAT CTC CTT TTC TGT GTC ACA
TAG AAA GTG TGG TTG GAC CGG TAG CGT CTA GAG GAA AAG ACA CAG TGT
I   F   H   T   M   L   A   I   A   D   L   L   F   C   V   T>

      490      500      510      520      530
*   *   *   *   *   *   *   *   *   *
CTG CCA TTT AAG ATC GCC TAC CAT CTC AAT GGC AAC AAC TGG GTA TTT
GAC GGT AAA TTC TAG CGG ATG GTA GAG TTA CCG TTG TTG ACC CAT AAA
L   P   F   K   I   A   Y   H   L   N   G   N   N   W   V   F>

      540      550      560      570
*   *   *   *   *   *   *   *   *   *
GGC GAG GTC ATG TGC CGG ATC ACC ACG GTC GTT TTC TAC GGC AAC ATG
CCG CTC CAG TAC ACG GCC TAG TGG TGC CAG CAA AAG ATG CCG TTG TAC
G   E   V   M   C   R   I   T   T   V   V   F   Y   G   N   M>

580      590      600      610      620
*   *   *   *   *   *   *   *   *   *
TAC TGC GCT ATC CTG ATC CTC ACT TGC ATG GGC ATC AAC CGC TAC CTG
ATG ACG CGA TAG GAC TAG GAG TGA ACG TAC CCG TAG TTG GCG ATG GAC
Y   C   A   I   L   I   L   T   C   M   G   I   N   R   Y   L>

      630      640      650      660      670
*   *   *   *   *   *   *   *   *   *
GCC ACG GCT CAC CCT TTC ACA TAC CAG AAG CTG CCC AAA CGC AGC TTC
CGG TGC CGA GTG GGA AAG TGT ATG GTC TTC GAC GGG TTT GCG TCG AAG
A   T   A   H   P   F   T   Y   Q   K   L   P   K   R   S   F>

      680      690      700      710      720
*   *   *   *   *   *   *   *   *   *
TCC TTG CTC ATG TGT GGC ATA GTG TGG GTC ATG GTT TTC TTA TAC ATG
AGG AAC GAG TAC ACA CCG TAT CAC ACC CAG TAC CAA AAG AAT ATG TAC
S   L   L   M   C   G   I   V   W   V   M   V   F   L   Y   M>

      730      740      750      760      770
*   *   *   *   *   *   *   *   *   *
CTG CCC TTT GTC ATC CTG AAG CAG GAG TAC CAC CTC GTC CAC TCA GAG
GAC GGG AAA CAG TAG GAC TTC GTC CTC ATG GTG GAG CAG GTG AGT CTC
L   P   F   V   I   L   K   Q   E   Y   H   L   V   H   S   E>

      780      790      800      810
*   *   *   *   *   *   *   *   *   *
ATC ACC ACC TGC CAC GAT GTC GTC GAC GCG TGC GAG TCC CCA TCA TCC
TAG TGG TGG ACG GTG CTA CAG CAG CTG CGC ACG CTC AGG GGT AGT AGG
I   T   T   C   H   D   V   V   D   A   C   E   S   P   S   S>

820      830      840      850      860
*   *   *   *   *   *   *   *   *   *
TTC CGA TTC TAC TAC TTC GTC TCC TTA GCA TTC TTT GGG TTC CTC ATC
AAG GCT AAG ATG ATG AAG CAG AGG AAT CGT AAG AAA CCC AAG GAG TAG
F   R   F   Y   Y   F   V   S   L   A   F   F   G   F   L   I>

```

SUBSTITUTE SHEET ( rule 26 )



3/16

FIG. 1-3

```

      870      880      890      900      910
      *      *      *      *      *
CCG TTT GTG ATC ATC ATC TTC TGT TAC ACG ACT CTC ATC CAC AAA CTT
GGC AAA CAC TAG TAG TAG AAG ACA ATG TGC TGA GAG TAG GTG TTT GAA
P  F  V  I  I  I  F  C  Y  T  T  L  I  H  K  L>
      920      930      940      950      960
      *      *      *      *      *
AAA TCA AAG GAT CGG ATA TGG CTG GGC TAC ATC AAG GCC GTC CTC CTC
TTT AGT TTC CTA GCC TAT ACC GAC CCG ATG TAG TTC CGG CAG GAG GAG
K  S  K  D  R  I  W  L  G  Y  I  K  A  V  L  L>
      970      980      990      1000      1010
      *      *      *      *      *
ATC CTT GTG ATT TTC ACA ATT TGC TTT GCC CCC ACC AAC ATC ATA CTC
TAG GAA CAC TAA AAG TGT TAA ACG AAA CGG GGG TGG TTG TAG TAT GAG
I  L  V  I  F  T  I  C  F  A  P  T  N  I  I  L>
      1020      1030      1040      1050
      *      *      *      *      *
GTA ATC CAC CAT GCC AAC TAC TAC TAC CAC AAT ACC GAC AGC TTG TAC
CAT TAG GTG GTA CGG TTG ATG ATG ATG GTG TTA TGG CTG TCG AAC ATG
V  I  H  H  A  N  Y  Y  Y  H  N  T  D  S  L  Y>
1060      1070      1080      1090      1100
      *      *      *      *      *
TTT ATG TAT CTT ATT GCT CTG TGC CTG GGG AGC CTG AAT AGC TGC CTA
AAA TAC ATA GAA TAA CGA GAC ACG GAC CCC TCG GAC TTA TCG ACG GAT
F  M  Y  L  I  A  L  C  L  G  S  L  N  S  C  L>
      1100      1120      1130      1140      1150
      *      *      *      *      *
GAT CCA TTC CTT TAC TTT GTC ATG TCG AAA GTT GTA GAT CAG CTT AAT
CTA GGT AAG GAA ATG AAA CAG TAC AGC TTT CAA CAT CTA GTC GAA TTA
D  P  F  L  Y  F  V  M  S  K  V  V  D  Q  L  N>
      1160      1170      1180      1190      1200
      *      *      *      *      *
CCT TAG TCG GCA ATG GCA AGA CCA CTT TAG AGA CCA AGG AGA GAT ATC
GGA ATC AGC CGT TAC CGT TCT GGT GAA ATC TCT GGT TCC TCT CTA TAG
P  *  S  A  M  A  R  P  L  *  R  P  R  R  D  I>
      1210      1220
      *      *      *      *
TGG GAA GAC ATA CAT GCT TGG C
ACC CTT CTG TAT GTA CGA ACC G
W  E  D  I  H  A  W  X>

```

4/16

FIG. 2-1

10	20	30	40	50
* *	* *	* *	* *	* *
CCATATGCTA	ATATTTCTT	TCAATTACAG	GCATAAATGT	TTCAGACAAC
60	70	80	90	100
* *	* *	* *	* *	* *
TCAGCAAAGC	CAACCTTAAC	TATTAAGAGT	TTTAATGGGG	GTCCCCAAAA
110	120	130	140	150
* *	* *	* *	* *	* *
TACCTTTGAA	GAATTC----	---TACAAC	CTCCATGTGA	ATAATGCTAC
160	170	180	190	200
* *	* *	* *	* *	* *
CATGGGATAC	CTGAGAAGTT	CCTTAAGTAC	CAAAGTGATA	CCTGCCATCT
210	220	230	240	250
* *	* *	* *	* *	* *
ACATCCTGGT	GTTTGTGATT	GGTGTACCAG	CGAACATCGT	GACCCTGTGG
260	270	280	290	300
* *	* *	* *	* *	* *
AAACTCTCCT	CAAGGACCAA	ATCCATCTGT	CTGGTCATCT	TTCACACCAA
310	320	330	340	350
* *	* *	* *	* *	* *
CCTGGCCATC	GCGGATCTCC	TTTTCTGTGT	CACGCTGCCG	TTTAAGATC-
360	370	380	390	400
* *	* *	* *	* *	* *
-CCTACCATC	TCAATGGCAA	CAACTGGGTA	TTTGGCGAGG	TCATGTGCCG
410	420	430	440	450
* *	* *	* *	* *	* *
GATCACCACG	GTCGTTTTCT	ACGGCAACAT	GTACTGCGCT	A---TCCTGA
460	470	480	490	500
* *	* *	* *	* *	* *
TCCTCACCTG	CATGGGCATC	AACCGCTACC	TGGCCACGGC	TCACCCTTTC
510	520	530	540	550
* *	* *	* *	* *	* *
ACATACCAGA	AGCTGCCCAA	ACGCAGCTTC	TCCATGCTCA	TGTGTGGCAT
560	570	580	590	600
* *	* *	* *	* *	* *
GGTGTGGGTC	ATGGTTTTCT	TATACATGCT	GCCCTTTGTC	ATCC---AAG
610	620	630	640	650
* *	* *	* *	* *	* *
CAGGAGTACC	ACCTCGTCCA	CTCCGAGATC	ACCACCTGCC	ACGATGTCGT

SUBSTITUTE SHEET ( rule 26 )

5/16

FIG. 2-2

660	670	680	690	700
* *	* *	* *	* *	* *
CGACGCGTGC	GANTCCCCAT	CATCCTTCCG	ATTCTACTAC	TTCGTCTCCT
710	720	730	740	750
* *	* *	* *	* *	* *
TAGCATTCTT	TGGGTTTCCTC	ATCCCGTTTG	TGATCATCAT	CTTCTGTTAC
760	770	780	790	800
* *	* *	* *	* *	* *
ACGACTCTCA	TCCACAAACT	TAAATCAAAA	GATCNGATAT	GGCTGGGCTA
810	820	830	840	850
* *	* *	* *	* *	* *
CATCAAGGCC	GTCCTCCTCA	TCCTTGTGAA	TTTCACCATC	TGCTTCCCCC
860	870	880	890	900
* *	* *	* *	* *	* *
CCACCAAG--	----GATATC	TGGGAAGACG	TACATGCTTG	GCTGACTTGT
910	920	930	940	950
* *	* *	* *	* *	* *
GCATGGCACC	ATCAGCTCAA	TTTTTAATTT	TTTAATTTTA	ATTTAATTTA
960	970	980	990	1000
* *	* *	* *	* *	* *
ATTTTATGTT	TTTGAGACAG	AGCCTCACTG	TGTAGTCCTG	GCTGGCCTGG
1010	1020	1030	1040	1050
* *	* *	* *	* *	* *
CTGGTTCTCT	ATTTAGACCA	GGTTAGCCTT	GAATCACAG	AGATCTGCCT
1060	1070	1080	1090	1100
* *	* *	* *	* *	* *
GCTTCTGCCT	CCCAAGTGCT	GGGTTCAACC	AGGTCTGGCA	AGCGCTCCAT
1110	1120			
* *	* *			
TTTTCAGCTC	CTCTGCAACA	GTGC		

6/16

FIG. 3-1

```

      10      20      30      40
      *      *      *      *      *      *      *      *
TGC TCC ATG ATT TTA CAG ATT TCA TAA CGT TTA AGA GAC GGG ACT CAG
ACG AGG TAC TAA AAT GTC TAA AGT ATT GCA AAT TCT CTG CCC TGA GTC
C  S  M  I  L  Q  I  S  *  R  L  R  D  G  T  Q>
50      60      70      80      90
      *      *      *      *      *      *      *      *
GTC ATC AAA ATG AAA GCC CTC ATC TTT GCA GCT GCT GGC CTC CTG CTT
CAG TAG TTT TAC TTT CGG GAG TAG AAA CGT CGA CGA CCG GAG GAC GAA
V  I  K  M  K  A  L  I  F  A  A  A  G  L  L  L>
100     110     120     130     140
      *      *      *      *      *      *      *      *
CTG TTG CCC ACT TTT TGT CAG AGT GGC ATG GAA AAT GAT ACA AAC AAC
GAC AAC GGG TGA AAA ACA GTC TCA CCG TAC CTT TTA CTA TGT TTG TTG
L  L  P  T  F  C  Q  S  G  M  E  N  D  T  N  N>
150     160     170     180     190
      *      *      *      *      *      *      *      *
TTG GCA AAG CCA ACC TTA CCC ATT AAG ACC TTT CGT GGA GCT CCC CCA
AAC CGT TTC GGT TGG AAT GGG TAA TTC TGG AAA GCA CCT CGA GGG GGT
L  A  K  P  T  L  P  I  K  T  F  R  G  A  P  P>
200     210     220     230     240
      *      *      *      *      *      *      *      *
AAT TCT TTT GAA GAG TTC CCC TTT TCT GCC TTG GAA GGC TGG ACA GGA
TTA AGA AAA CTT CTC AAG GGG AAA AGA CGG AAC CTT CCG ACC TGT CCT
N  S  F  E  E  F  P  F  S  A  L  E  G  W  T  G>
250     260     270     280
      *      *      *      *      *      *      *      *
GCC ACG ATT ACT GTA AAA ATT AAG TGC CCT GAA GAA AGT GCT TCA CAT
CGG TGC TAA TGA CAT TTT TAA TTC ACG GGA CTT CTT TCA CGA AGT GTA
A  T  I  T  V  K  I  K  C  P  E  E  S  A  S  H>
290     300     310     320     330
      *      *      *      *      *      *      *      *
CTC CAT GTG AAA AAT GCT ACC ATG GGG TAC CTG ACC AGC TCC TTA AGT
GAG GTA CAC TTT TTA CGA TGG TAC CCC ATG GAC TGG TCG AGG AAT TCA
L  H  V  K  N  A  T  M  G  Y  L  T  S  S  L  S>
340     350     360     370     380
      *      *      *      *      *      *      *      *
ACT AAA CTG ATA CCT GCC ATC TAC CTC CTG GTG TTT GTA GTT GGT GTC
TGA TTT GAC TAT GGA CGG TAG ATG GAG GAC CAC AAA CAT CAA CCA CAG
T  K  L  I  P  A  I  Y  L  L  V  F  V  V  G  V>
390     400     410     420     430
      *      *      *      *      *      *      *      *
CCG GCC AAT GCT GTG ACC CTG TGG ATG CTT TTC TTC AGG ACC AGA TCC
GGC CGG TTA CGA CAC TGG GAC ACC TAC GAA AAG AAG TCC TGG TCT AGG
P  A  N  A  V  T  L  W  M  L  F  F  R  T  R  S>

```

SUBSTITUTE SHEET ( rule 26 )

7/16

FIG. 3-2

```

      440      450      460      470      480
      *      *      *      *      *      *      *      *
    ATC TGT ACC ACT GTA TTC TAC ACC AAC CTG GCC ATT GCA GAT TTT CTT
    TAG ACA TGG TGA CAT AAG ATG TGG TTG GAC CGG TAA CGT CTA AAA GAA
    I  C  T  T  V  F  Y  T  N  L  A  I  A  D  F  L>

      490      500      510      520
      *      *      *      *      *      *      *
    TTT TGT GTT ACA TTG CCC TTT AAG ATA GCT TAT CAT CTC AAT GGG AAC
    AAA ACA CAA TGT AAC GGG AAA TTC TAT CGA ATA GTA GAG TTA CCC TTG
    F  C  V  T  L  P  F  K  I  A  Y  H  L  N  G  N>

    530      540      550      560      570
      *      *      *      *      *      *      *
    AAC TGG GTA TTT GGA GAG GTC CTG TGC CGG GCC ACC ACA GTC ATC TTC
    TTG ACC CAT AAA CCT CTC CAG GAC ACG GCC CGG TGG TGT CAG TAG AAG
    N  W  V  F  G  E  V  L  C  R  A  T  T  V  I  F>

      580      590      600      610      620
      *      *      *      *      *      *      *
    TAT GGC AAC ATG TAC TGC TCC ATT CTG CTC CTT GCC TGC ATC AGC ATC
    ATA CCG TTG TAC ATG ACG AGG TAA GAC GAG GAA CGG ACG TAG TCG TAG
    Y  G  N  M  Y  C  S  I  L  L  L  A  C  I  S  I>

      630      640      650      660      670
      *      *      *      *      *      *      *
    AAC CGC TAC CTG GCC ATC GTC CAT CCT TTC ACC TAC CGG GGC CTG CCC
    TTG GCG ATG GAC CGG TAG CAG GTA GGA AAG TGG ATG GCC CCG GAC GGG
    N  R  Y  L  A  I  V  H  P  F  T  Y  R  G  L  P>

      680      690      700      710      720
      *      *      *      *      *      *      *
    AAG CAC ACC TAT GCC TTG GTA ACA TGT GGA CTG GTG TGG GCA ACA GTT
    TTC GTG TGG ATA CGG AAC CAT TGT ACA CCT GAC CAC ACC CGT TGT CAA
    K  H  T  Y  A  L  V  T  C  G  L  V  W  A  T  V>

      730      740      750      760
      *      *      *      *      *      *      *
    TTC TTA TAT ATG CTG CCA TTT TTC ATA CTG AAG CAG GAA TAT TAT CTT
    AAG AAT ATA TAC GAC GGT AAA AAG TAT GAC TTC GTC CTT ATA ATA GAA
    F  L  Y  M  L  P  F  F  I  L  K  Q  E  Y  Y  L>

    770      780      790      800      810
      *      *      *      *      *      *      *
    GTT CAG CCA GAC ATC ACC ACC TGC CAT GAT GTT CAC AAC ACT TGC GAG
    CAA GTC GGT CTG TAG TGG TGG ACG GTA CTA CAA GTG TTG TGA ACG CTC
    V  Q  P  D  I  T  T  C  H  D  V  H  N  T  C  E>

      820      830      840      850      860
      *      *      *      *      *      *      *

```

8/16

## FIG. 3-3

```

      870      880      890      900      910
      *      *      *      *      *
GGA TTC TTA ATT CCA TTT GTG CTT ATC ATC TAC TGC TAT GCA GCC ATC
CCT AAG AAT TAA GGT AAA CAC GAA TAG TAG ATG ACG ATA CGT CGG TAG
G   F   L   I   P   F   V   L   I   I   Y   C   Y   A   A   I>
      920      930      940      950      960
      *      *      *      *      *
ATC CGG ACA CTT AAT GCA TAC GAT CAT AGA TGG TTG TGG TAT GTT AAG
TAG GCC TGT GAA TTA CGT ATG CTA GTA TCT ACC AAC ACC ATA CAA TTC
I   R   T   L   N   A   Y   D   H   R   W   L   W   Y   V   K>
      970      980      990      1000
      *      *      *      *      *
GCG AGT CTC CTC ATC CTT GTG ATT TTT ACC ATT TGC TTT GCT CCA AGC
CGC TCA GAG GAG TAG GAA CAC TAA AAA TGG TAA ACG AAA CGA GGT TCG
A   S   L   L   I   L   V   I   F   T   I   C   F   A   P   S>
1010      1020      1030      1040      1050
      *      *      *      *      *
AAT ATT ATT CTT ATT ATT CAC CAT GCT AAC TAC TAC TAC AAC AAC ACT
TTA TAA TAA GAA TAA TAA GTG GTA CGA TTG ATG ATG ATG TTG TTG TGA
N   I   I   L   I   I   H   H   A   N   Y   Y   Y   N   N   T>
1060      1070      1080      1090      1100
      *      *      *      *      *
GAT GGC TTA TAT TTT ATA TAT CTC ATA GCT TTG TGC CTG GGT AGT CTT
CTA CCG AAT ATA AAA TAT ATA GAG TAT CGA AAC ACG GAC CCA TCA GAA
D   G   L   Y   F   I   Y   L   I   A   L   C   L   G   S   L>
1110      1120      1130      1140      1150
      *      *      *      *      *
AAT AGT TGC TTA GAT CCA TTC CTT TAT TTT CTC ATG TCA AAA ACC AGA
TTA TCA ACG AAT CTA GGT AAG GAA ATA AAA GAG TAC AGT TTT TGG TCT
N   S   C   L   D   P   F   L   Y   F   L   M   S   K   T   R>
1160      1170      1180      1190      1200
      *      *      *      *      *
AAT CAC TCC ACT GCT TAC CTT ACA AAA TAG TGA AAT GAT CTT AGA GAA
TTA GTG AGG TGA CGA ATG GAA TGT TTT ATC ACT TTA CTA GAA TCT CTT
N   H   S   T   A   Y   L   T   K   *   *   N   D   L   R   E>
1210      1220
      *      *      *      *
CAA GGA CAG CCA TCA CAG AGA ACG
GTT CCT GTC GGT AGT GTC TCT TGC
Q   G   Q   P   S   Q   R   T>

```

SUBSTITUTE SHEET ( rule 26 )

9/16

FIG. 4-1

10	20	30	40	50
* *	* *	* *	* *	* *
-ACAGGCATG	GAAAATGATA	CAAACAACCTT	GGCAAAGCCA	ACCTTACCCA
60	70	80	90	100
* *	* *	* *	* *	* *
TTAAGACCTT	TCGTGGAGCT	CCCCCAAATT	CTTTTGAAGA	GTTCCCCTTT
110	120	130	140	150
* *	* *	* *	* *	* *
TCTGCCTTGG	AAGGCTGGAC	AGGAGCCACG	ATTACTGTAA	AAATTAAGTG
160	170	180	190	200
* *	* *	* *	* *	* *
CCCTGAAGAA	AGTGCTTCAC	ATCTCCATGT	GAAAAATGCT	ACCATGGGGT
210	220	230	240	250
* *	* *	* *	* *	* *
ACCTGACCAG	CTCCTTAAGT	ACTAACTGA	TACCTGCCAT	CTACCTCCTG
260	270	280	290	300
* *	* *	* *	* *	* *
GTGTTTGTAG	TTGGTGTCCC	GGCCAATGCT	GTGACCCTGT	GGATGCTTTT
310	320	330	340	350
* *	* *	* *	* *	* *
CTTCAGGACC	AGATCCATCT	GTACCACTGT	ATTCTACACC	AACCTGGCCA
360	370	380	390	400
* *	* *	* *	* *	* *
TTGCAGATTT	TCTTTTTTGT	GTTACATTGC	CCTTTAAGAT	AGCTTATCAT
410	420	430	440	450
* *	* *	* *	* *	* *
CTCAATGGGA	ACAACTGGGT	ATTTGGAGAG	GTCCTGTGCC	GGGCCACCAC
460	470	480	490	500
* *	* *	* *	* *	* *
AGTCATCTTC	TATGGCAACA	TGTACTGCTC	CATTCTGCTC	CTTGCCTGCA
510	520	530	540	550
* *	* *	* *	* *	* *
TCAGCATCAA	CCGCTACCTG	GCCATCGTCC	ATCCTTTTAC	CTACCGGGGC
560	570	580	590	600
* *	* *	* *	* *	* *
CTGCCCCAAGC	ACACCTATGC	CTTGGAACA	TGTGGACTGG	TGTGGGCAAC
AGTTTCTTA TATATGCTGC CATTTTTCAT ACTGAAGCAG GAATATTATC				

SUBSTITUTE SHEET (rule 26)

10/16

## FIG. 4-2

660	670	680	690	700
* *	* *	* *	* *	* *
TTGTTGAGCC	AGACATCACC	ACCTGCCATG	ATGTTACAAA	CACCTGCGAG
710	720	730	740	750
* *	* *	* *	* *	* *
TCCTCATCTC	CCTTCCAAC	CTATTACTTC	ATCTCCTTGG	CATTCTTTGG
760	770	780	790	800
* *	* *	* *	* *	* *
ATTCTTAATT	CCATTTGTGC	TTATCATCTA	CTGCTATGCA	GCCATCATCC
810	820	830	840	850
* *	* *	* *	* *	* *
GGACACTTAA	TGCATACGAT	CATAGATGGT	TGTGGTATGT	TAAGGCGAGT
860	870	880	890	900
* *	* *	* *	* *	* *
CTCCTCATCC	TTGTGATTTT	TACCATTTC	TTTGCTCCAA	GCAATATTAT
910	920	930	940	950
* *	* *	* *	* *	* *
TCTTATTATT	CACCATGCTA	ACTACTACTA	CAACAACACT	GATGGCTTAT
960	970	980	990	1000
* *	* *	* *	* *	* *
ATTTTATATA	TCTCATAGCT	TTGTGCCTGG	GTAGTCTTAA	TAGTTGCTTA
1010	1020	1030	1040	1050
* *	* *	* *	* *	* *
GATCCATTCC	TTTATTTTCT	CATGTCAAAA	ACCAGAAATC	ACTCCACTGC
1060	1070	1080	1090	1100
* *	* *	* *	* *	* *
TTACCTTACA	AAATAGTGAA	ATGATCTTAG	AGAACAAGGA	CAGCCATCAC

AGA

SUBSTITUTE SHEET ( rule 26 )



FIG. 1

hPAR3 1 LTFAAAGLLLLP TFCQSGMENDINNLA KP TLPIK / TFRGAPPN SFEFFPFSALEGWTGATITVKIKC PEESASHLHVKNATMG  
hPAR1 2 R LLLVAACFSLCGP LLSARTRARPESKATNATLDPR / SFLLRNPNDKYEPFWEDEEKNE SGL TEYRLVSINKSSPLQKQLPAFISEDASG  
hPAR2 3 SAUWLLGAAAILLA ASLSCSGTIQG TNRSSKGR / SLIGKYDGTSHVTGKGTIV ETVFSVDEFSAS

hPAR3 4 SLSSTKLIPAIYLLV FVVGV PANAVTLWMLFFRTR SICITVFTYNLAIA DFLFCVTL PFKIAYHLNGNNWVGEVLCRATTVIFVGNMYCSILLACISINRYLAI  
hPAR1 5 SSWLLTLFVPSVYTG VVVS LPLNIMAI VVFI LKMKVKKPAVVYMLHLATADVLFVSVLPFKISYYFSGSDQFGSEL CRFVTAAFCNMYASILLMTVISIDRFLAV  
hPAR2 6 SKLTTVFLPIVYTI VIVFVVG LPSNGMALWVFLFRTKKKHPAVIYMANLALADLLSVIWFPLKIAYHIIHGNNWIYGEALCNVLIGFFYGNMYCSILFMTCLSVQRYWVI

hPAR3 7 PFTYRGLPKHTYALVTCGLVWATVFLYMLPFFILKQEYVLVQPDITTC HDVHNTCESSPFQLYYFISLAF FGLIPFVLIICYAAIIRTLNA YDHRWLWYV  
hPAR1 8 PMQSLSWRTLGRASF TCI LAIWALAIAGVVPVLVKEQTIQVPLNITTC HDVNETLLEG YYAYYFSAFSAVFFVPLIISTVCVCSIIIRCLSSSAVANRSKK SRAL  
hPAR2 9 PMGHSRKKANIAIGI SLAIWLLILLVTIPLVYVVKQTI FIPALNITTC HDVLPQELLVGD MFNYFLSLATGTVFLFPAFLTASAYVLMIRMLRSSAMDENSEKKRKRAI

hPAR3 10 SLLILVIFTICFAPSNIILIIHHANYYYNNI DGLYFIYLIALCLGSLNSCLDPFLYFLMSKTRNHSTAYLTK  
hPAR1 11 SAAVFCIFIICFGPTNVLLIAHYSFLSHTSTTEAAYFAYLLCVCVSSISSCIDPLIYYASSECQRYVYSILCCKESSDPSSYNSGQLMASKMDTCSSNLNNSIYKFL  
hPAR2 12 TVTVLAMYLCFTPSNLLLVVHY FLIKSQGQSHVYALYIVALCLSTLNSCIDPFVYVYVSHDFRDHAKNALLCRSVRTVKMQVSLTSKKHSRKSSSYSSSSTTVKTSY

FIG. 2

Hirud 1 ..DFEEIPEEYLQ  
hPAR3 2 ..TLPIK / TFRGAPPN SFEFFPFSALEGWTGA..  
hPAR1 3 ..TLDPR / SFLLRNPNDKYEPFWEDEEKNE SGL..  
hPAR2 4 ..SSKGR / SLIGKYDGTSHVTGKGTIVETVFSVD..

12/16

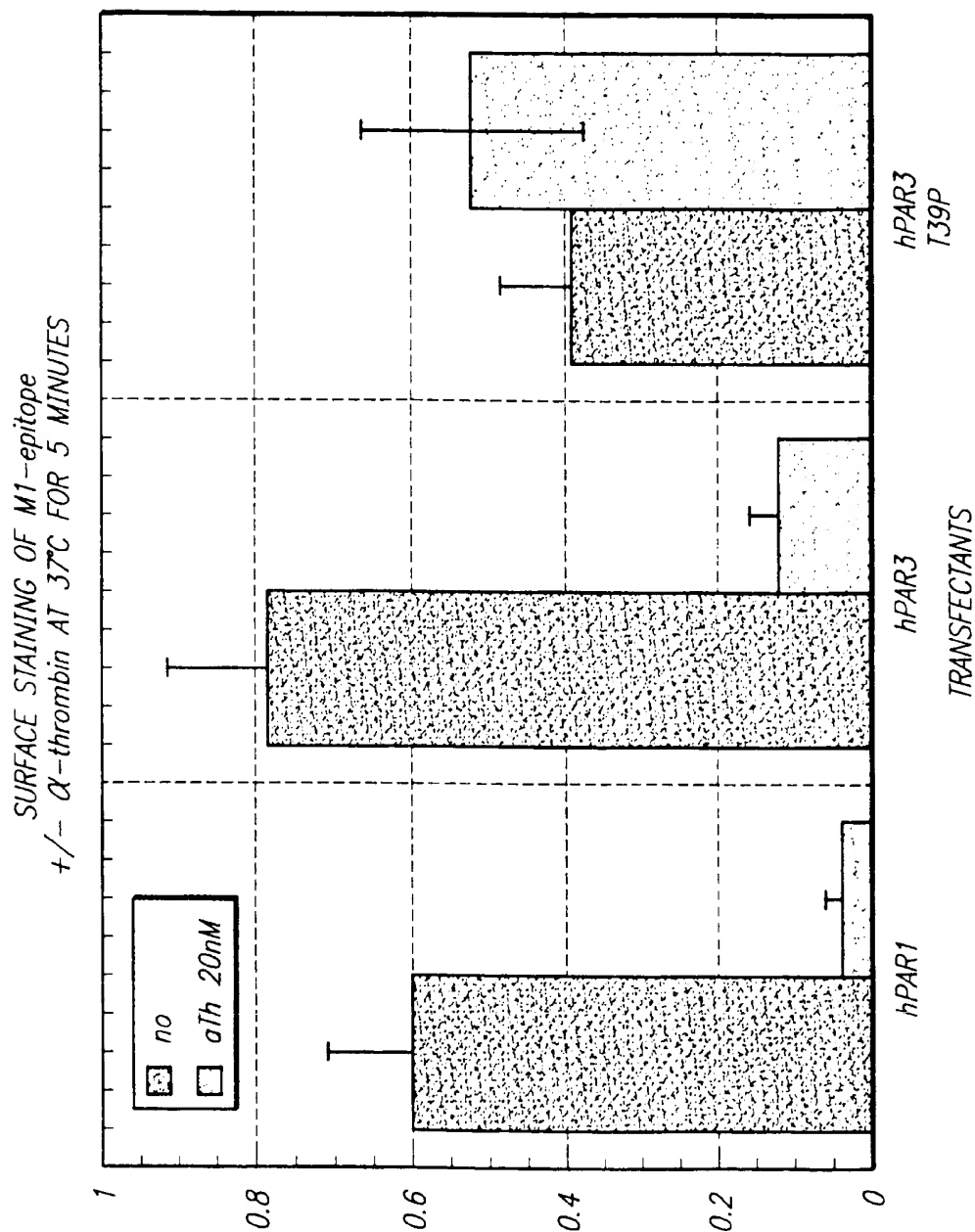
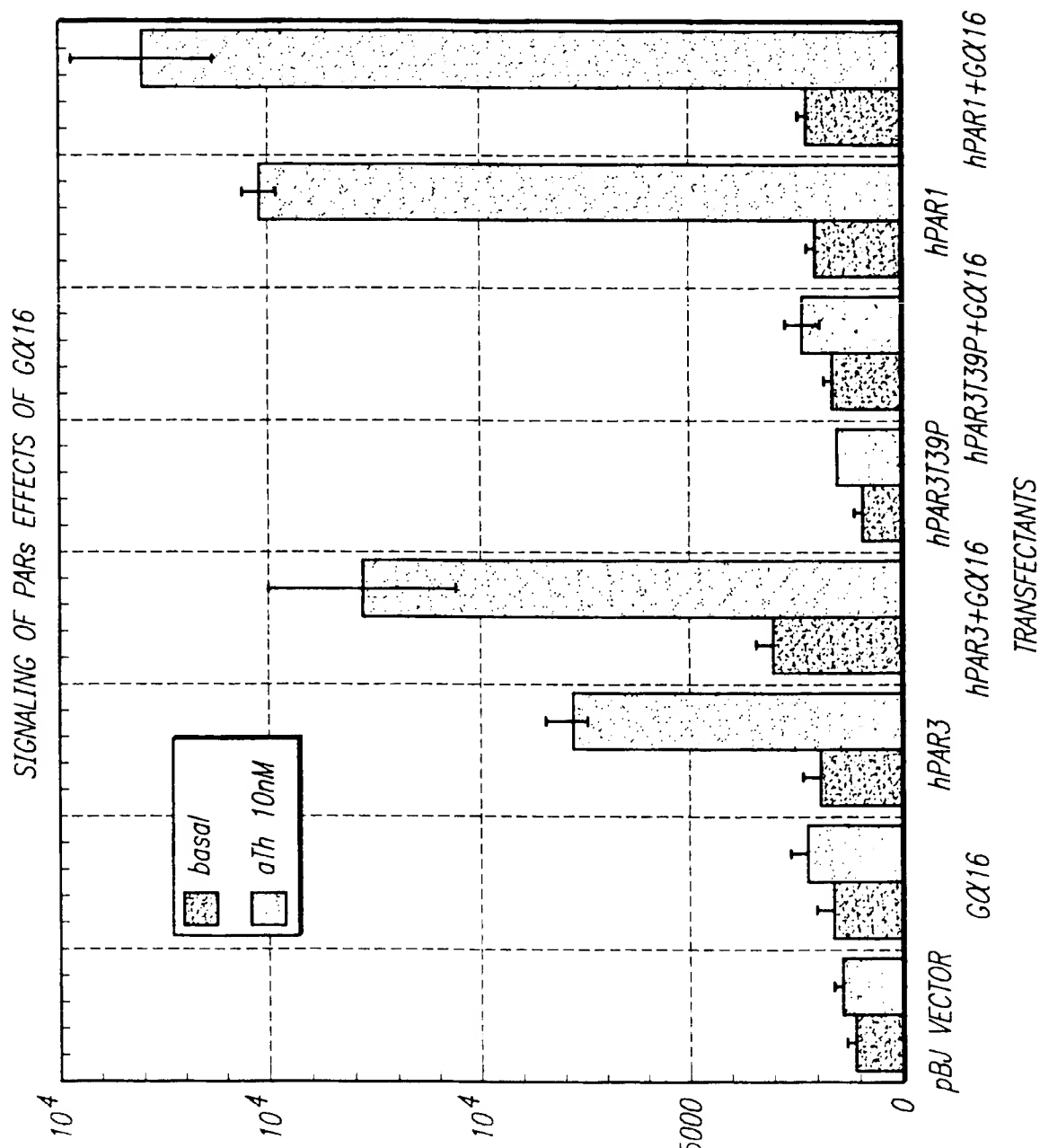


FIG. 6

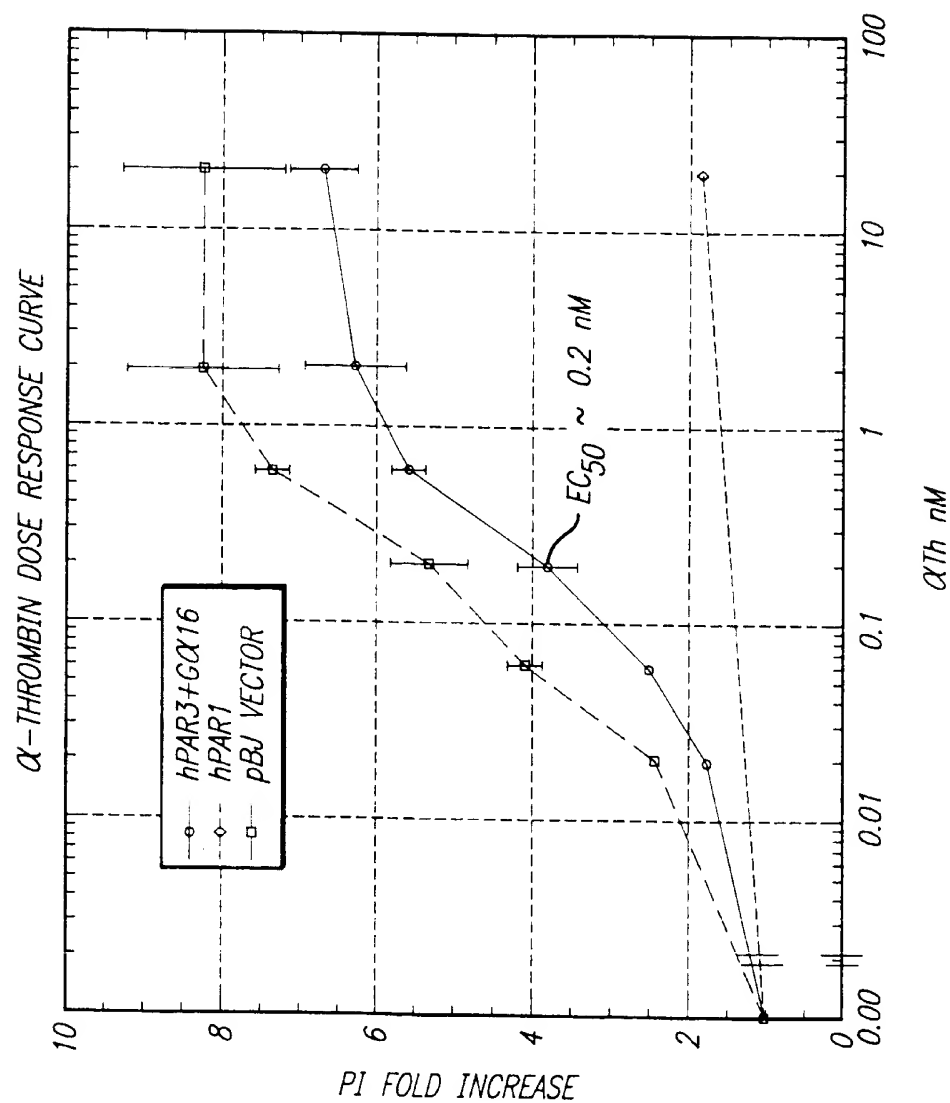
13/16

FIG. 7



14/16

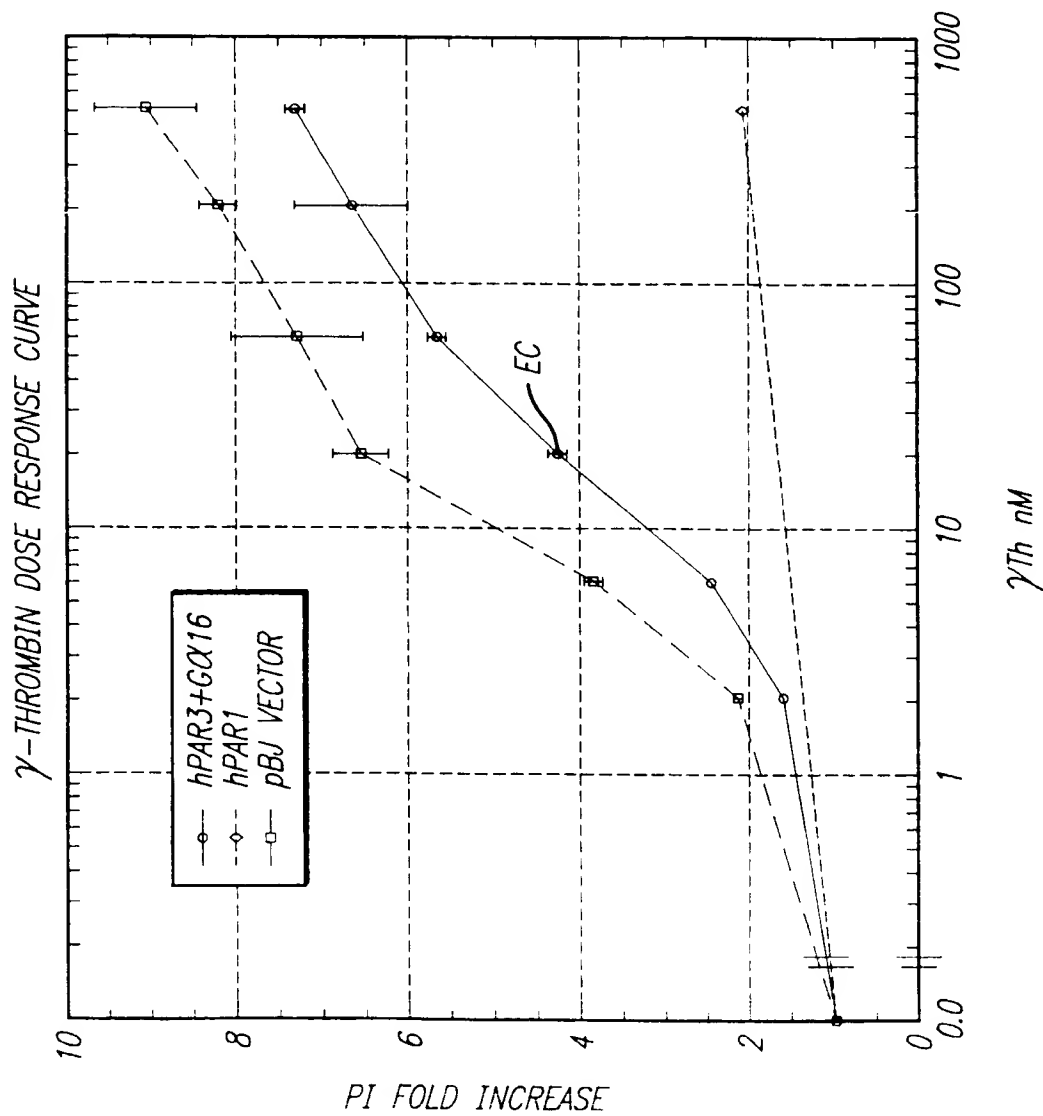
FIG. 8



SUBSTITUTE SHEET ( rule 26 )

15/16

FIG. 9



SUBSTITUTE SHEET (rule 26)

16/16

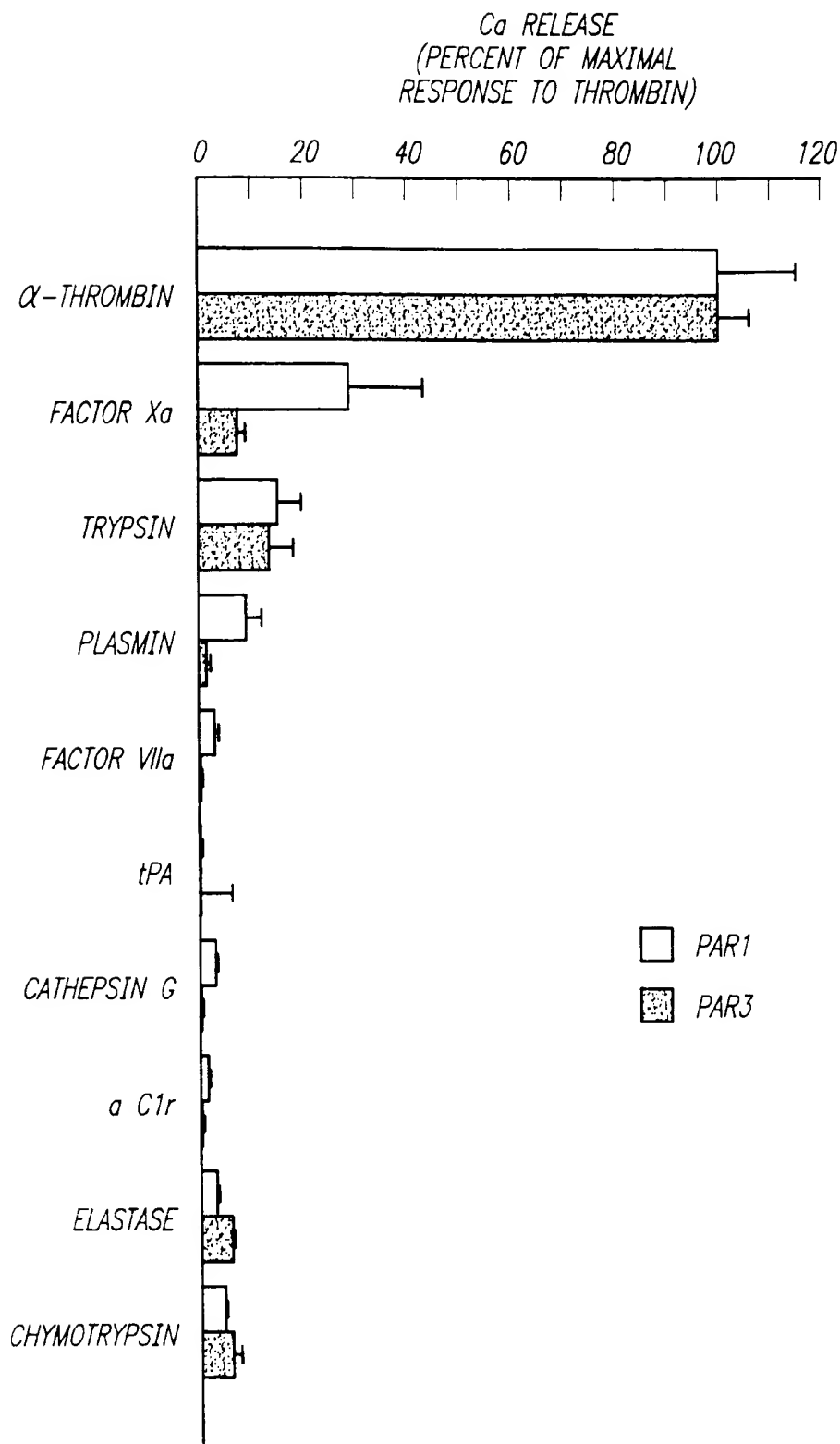


FIG. 10

SUBSTITUTE SHEET ( rule 26 )

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/19732

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : Please See Extra Sheet.

US CL : 435/7.2, 13, 69.1, 320.1, 325, 395; 514/1; 530/350; 536/23.5

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.2, 13, 69.1, 320.1, 325, 395; 514/1; 530/350; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P ----- A, P	ISHIHARA et al. Protease-activated receptor 3 is a second thrombin receptor in humans. Nature. 03 April 1997, Vol. 386, pages 502-506, especially figures 1-4.	1-13 ----- 14-18
X ----- Y	NYSTEDT et al. Molecular cloning of a potential proteinase activated receptor. Proc. Natl. Acad. Sci. USA. September 1994, vol. 91, pages 9208-9812, see especially Figures 2-6 and page 9208 (Materials and Methods).	1-2, 5, 9-13 ----- 14-17
X, E ----- Y, E	US 5,686,597 A (COLEMAN et al.) 11 November 1997 (11/11/97), espically columns 3-5, 7, and 10.	1-2, 5, 8-13, 17-18 ----- 14-16



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	
*O* document referring to an oral disclosure, use, exhibition or other means	

10 JANUARY 1998

23 FEB 1998

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No (703) 305 3230

Authorized officer

MICHAEL D. PAK

Telephone No (703) 308 0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/19732

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,256,766 A (COUGHLIN et al.) 26 October 1993 (23/10/93), see especially columns 4-5, 7-10, 14-16, and 18-22.	1-2, 5, 8-18

Form PCT/ISA/210 (continuation of second sheet)(July 1992)\*



# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/19732

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/19732

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

A61K 31/00, 35/00, 38/00; C07K 14/435, 14/705; C12N 5/10, 15/12; 15/63; G01N 33/53, 33/566

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN, BIOSCIENCE, BIOSIS, MEDLINE, CAPLUS, SCISEARCH, WPIDS

search terms: thrombin?(5a)receptor?, protease?(5a)receptor?, agonist?, antagonist?, therap?

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-16, drawn to a substantially pure DNA, a vector, an isolated protease-activated receptor 3, a substantially pure protein, a substantially pure polypeptide, a cell, an assay device, and a method of testing a candidate compound for agonist, a method of testing a candidate compound for antagonist and a method of testing a candidate compound with thrombin and platelets.

Group II, claim(s) 17, drawn to a therapeutic composition of agonist.

Group III, claim(s) 18-19, drawn to a therapeutic composition of antagonist.

Group IV, claim 20, drawn to a method of administering the therapeutic composition of antagonist.

The inventions listed as Groups do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons.

The main invention is Group I which is first product, first method of making the product, and the first method of using the product. Pursuant to 37 CFR 1.475(d), these claims are considered by the ISA/US to constitute the main invention, and none of the related groups II-IV correspond to the main invention.

The products of Group II and III, do not share the same or corresponding special technical feature with Group I, because they are drawn to products having materially different structures and functions, and each defines a separate invention over the art.

The methods of Group IV do not share the same or corresponding technical feature with Group I, because the methods have materially different process steps and are practiced for materially different purposes; and, the products of Group I is not used in or made by the method of Group IV; thus, each defines a separate invention over the art.

Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.